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RADIOACTIVE TRACER STUDY OF ENDOGENOUS METABOLISM

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June 1961

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FINAL REPORT ON PROJECT K-22

September 1, 1957 to January 1, 1961

RADIOACTIVE TRACER STUDY OF ENDOGENOUS METABOLISM

by

James M. Symons
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Cambridge, Massachusetts

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SUMMARY

This investigation covered studies on (1) the accumulation of volatile solids in extended sludge aeration activated sludge systems grown on sodium acetate, (2) the rates of synthesis and degradation of proteins, carbohydrates, and fats in extended sludge aeration activated sludge.

It was found that for extended sludge aeration (long sludge retention time) activated sludge operating either as a conventional activated sludge plant or as a uniform concentration activated sludge plant, volatile solids continually accumulated in the system. For systems grown on sodium acetate and loaded at a rate of 40 pounds of BOD_5 per 1000 cu. ft. of aeration capacity per day, volatile solids accumulated at a rate of 21.3 per cent of the BOD_5 removed for the conventional plant and 16.9 per cent of the BOD_5 removed for the uniform concentration plant. These data were taken for 20 weeks of operation.

By the use of radioactive tracers both conventional and uniform concentration activated sludge systems grown on sodium acetate, glucose, and glycine were shown to have high quantities of daily synthesis. This was balanced by daily loss of mass through endogenous respiration. Some of the synthesized mass was relatively biologically inert, however, as was shown by declining specific activities in the sludge

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fractions after feeding was stopped. Further studies showed that the inert mass accumulating in the system was about 50 per cent carbohydrate, 44 per cent nitrogen containing (measured as protein) and 6 per cent fats. These ratios were fairly constant for the three substrates studied and for both types of activated sludge systems studied. The steady accumulation of inert mass which contains some nitrogen meant that about 0.5 per cent of the BOD_5 being treated by activated sludge must be supplied as nitrogen if normal operation was to be maintained.

For the degradable substrates studied, o-, m-, p-amino Na benzoate and o-, m-, p-nitro Na benzoate, the general mechanism of degradation was the same, the nitrogen was released to solution as ammonia nitrogen during metabolism in all cases. In both cases the meta form of the compound was the most difficult to degrade. 3, 5-dinitro Na benzoate and 2, 4, 6-trinitro Na benzoate were found to be undegradable by activated sludge.

RADIOACTIVE TRACER STUDY OF ENDOGENOUS METABOLISM

J. M. Symons, D. R. Washington, R. E. McKinney, and
R. E. Speece.

During the past four or five years a new modification of the activated sludge process has become very popular, particularly for industries and small domestic sewage systems. The significant feature of this modification which makes it different from other activated sludge systems is that the incoming waste is rapidly dispersed throughout the entire aeration tank. This means that the concentration of organic matter in solution is uniform throughout the aeration tank at any time. For this reason this modification will be called "uniform concentration" activated sludge.

In any activated sludge system operating on a completely degradable feed, the actual net accumulation of volatile solids in the system is the difference between the conversion of the incoming food to new cells minus the biological destruction of mass by the process of endogenous respiration. In equation form:

$$\text{Accumulation} = \text{Synthesis} - \text{Endogenous Respiration} \qquad (1)$$

$$A = aF - bM_d \qquad (2)$$

where A = the pounds of volatile solids increase per day
a = the fraction of incoming food converted to new cells
F = the food added to the system per day, measured in pounds of COD, pounds of ultimate BOD, or pounds of five day BOD. "a" is different depending on the units of "F"
b = the rate of degradable mass destruction by endogenous respiration under fed conditions
M_d = the quantity of volatile degradable mass in the system at any time, in pounds.

When uniform concentration activated sludge was first studied some investigators felt that by providing an ample sludge aeration period (long sludge retention time) the quantity of mass destroyed by endogenous respiration could be made to equal the quantity of new synthesis each day and the system would operate with

"A" = 0, thus eliminating the necessity for sludge disposal facilities. Further investigation showed, however, that a portion of the new cells produced each day were resistant to degradation by endogenous respiration and therefore were considered biologically inert. This being the case, this type of activated sludge system, called "extended sludge aeration," could not operate with "A" = 0, but the minimum "A" would be equal to M_i (inert mass) produced per day.

Note that an extended sludge aeration activated sludge plant does not have to be a uniform concentration activated sludge plant and a uniform concentration activated sludge plant does not have to be operated with an extended sludge aeration period.

PURPOSE

The major purpose of this investigation was to study the quantity and composition of the volatile solids accumulating in extended sludge aeration activated sludge plants operating with a minimum of sludge wasting. Other phases of the study concerned the rate of turnover of sludge in an extended sludge aeration activated sludge system and ability of activated sludge to metabolize various organic nitrogen compounds.

APPROACH

In order to study the accumulation of volatile solids under different environmental conditions activated sludges were grown, in duplicate, on three different substrates. The 1.5 liter pilot plants were operated at two different leading schedules, (1) continuously fed simulating uniform concentration activated sludge and (2) batch fed simulating conventional activated sludge with a high concentration of organics in solution at the start of the aeration period which decreases as the food is metabolised. The three substrates used were sodium acetate, glucose, and glycine, to represent the three major classes of organic compounds; fatty acids, carbohydrates, and protein. These substrates were used as they were felt to be the simplest members of the major organic categories mentioned above. All units were fed organics equivalent to 40 lbs of COD/ 1000 Ft^3 of pilot plant capacity/day. No sludge was wasted from these systems except for sampling and as solids in the effluent.

For each substrate studied the investigation fell into three phases: one, the completion of week long carbon balances to find the per cent of the fed carbon which left the system each day as CO_2 and the per cent which accumulated in the system as volatile solids; two, the introduction of radioactive substrate to the system to find the uptake of radioactive C_{14} into the protein, fat, and carbohydrate fractions of the sludge with time; and three, the stopping of feeding and the measurement of the decline of the specific activity in the protein, fat, and carbohydrate fractions of the sludge with time and without feeding was also determined. A final study was the measurement of the production of radioactive CO_2 with time as feeding a non-radioactive substrate was continued.

A schematic diagram of the pilot plants used in this investigation with the gas trains used for scrubbing the CO_2 from the influent air and capturing the CO_2 released by the microorganisms are shown in Figures 1 and 2 and pictured in Figure 3. A schematic diagram of the apparatus used to burn the radioactive carbon samples to CO_2 is shown in Figure 4. The equipment used to count the radioactive $BaCO_3$ is pictured in Figure 5. Detailed descriptions of the procedures for the operation of the pilot plants, completion of carbon balances, and isolation, measurement, and counting for the protein, fat, and carbohydrate fractions of the sludges were described in detail in the February 8, 1959, Progress Report on Project K-22, a portion of which is appended to this report. The phase of the project concerning the metabolism of organic nitrogen by activated sludge is described in the last section of this report.

THEORETICAL CONSIDERATIONS

In a conventionally operated activated sludge system, metabolism of the waste by activated sludge organisms is accompanied by a synthesis of new sludge organisms with a comparatively small loss of organic mass due to autolysts. In these systems a portion of the return sludge is continually wasted in order to maintain a fairly constant level of suspended solids. If the system operation is altered such that synthesized sludge organisms can accumulate, the food to microorganism ratio decreased. Under these conditions the organisms have increasingly less food per organism and as a result, must rely more and more upon the organic matter within the sludge as a source of energy, that is to say the organisms spend an increasingly substantial portion of the retention period in endogenous

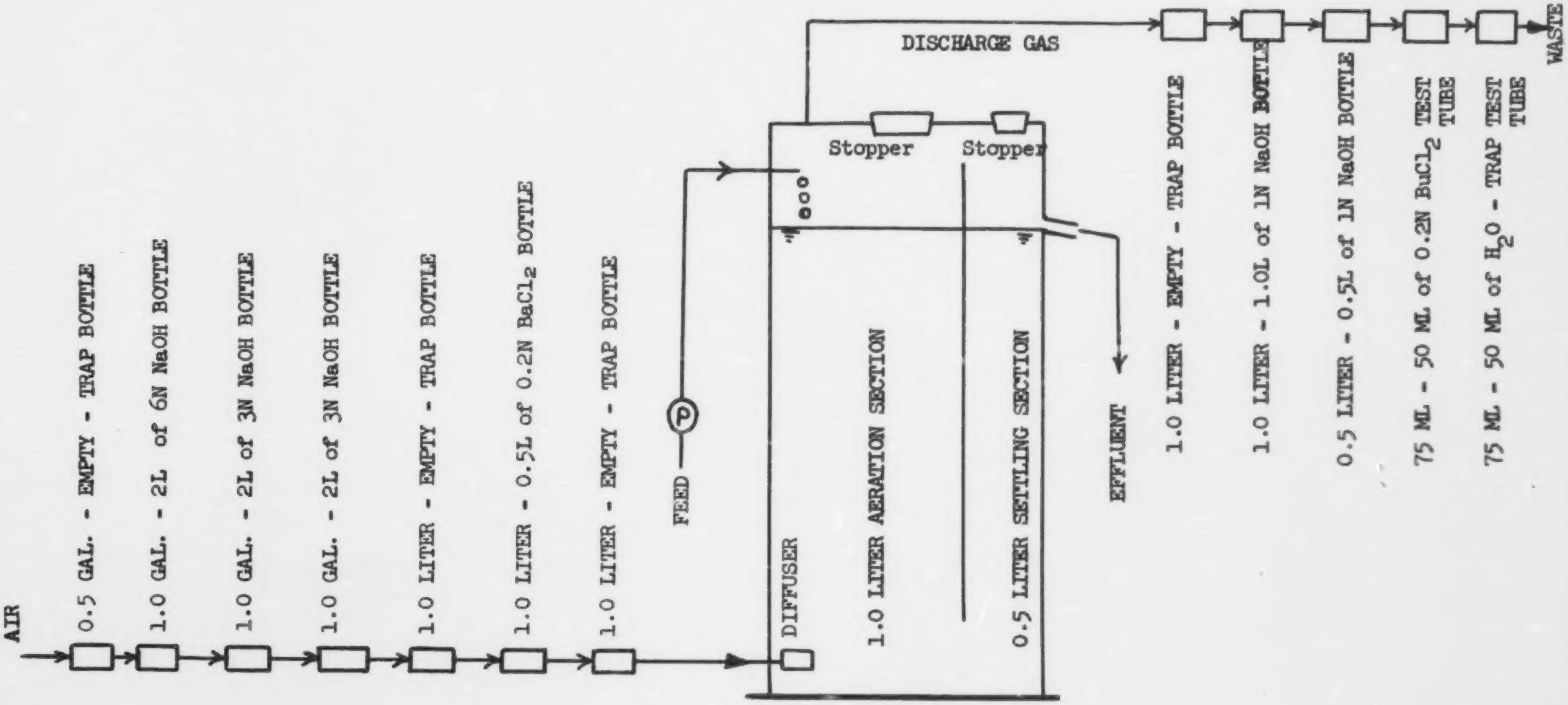

AIR
0.5 GAL. - EMPTY - TRAP BOTTLE
1.0 GAL. - 2L of 6N NaOH BOTTLE
1.0 GAL. - 2L of 3N NaOH BOTTLE
1.0 GAL. - 2L of 3N NaOH BOTTLE
1.0 LITER - EMPTY - TRAP BOTTLE
1.0 LITER - 0.5L of 0.2N BaCl2 BOTTLE
1.0 LITER - EMPTY - TRAP BOTTLE
FEED
P
DIFFUSER
1.0 LITER AERATION SECTION
0.5 LITER SETTLING SECTION
Stopper
Stopper
EFFLUENT
DISCHARGE GAS
1.0 LITER - EMPTY - TRAP BOTTLE
1.0 LITER - 1.0L of 1N NaOH BOTTLE
0.5 LITER - 0.5L of 1N NaOH BOTTLE
75 ML - 50 ML of 0.2N BuCl2 TEST TUBE
75 ML - 50 ML of H2O - TRAP TEST TUBE
WASTE

FIG 1 - CROSS SECTION SCHEMATIC DIAGRAM OF : CONTINUOUS FLOW PILOT PLANT

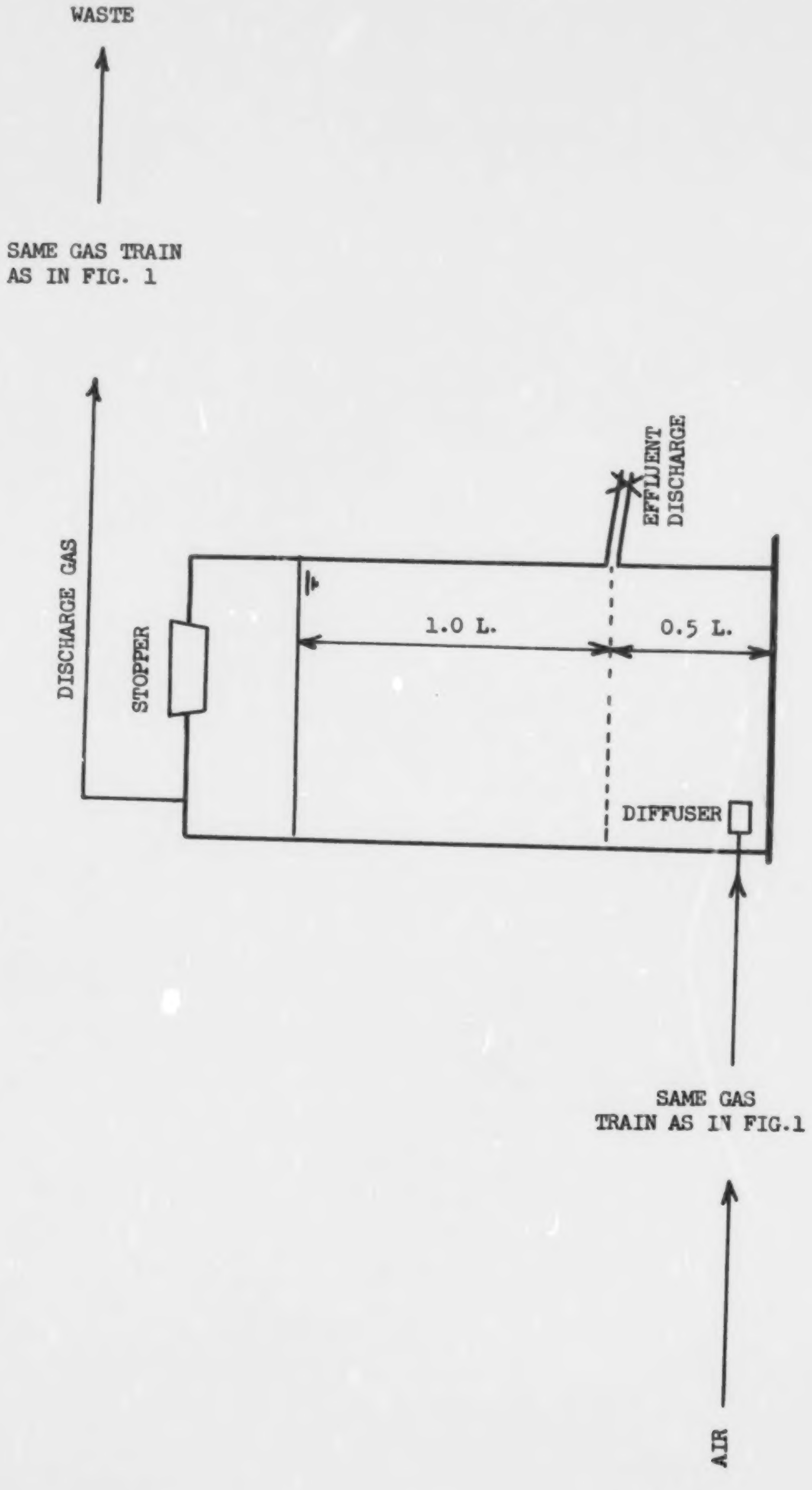

WASTE
SAME GAS TRAIN AS IN FIG. 1
DISCHARGE GAS
STOPPER
EFFLUENT DISCHARGE
1.0 L.
0.5 L.
DIFFUSER
SAME GAS TRAIN AS IN FIG.1
AIR

FIG. 2 - CROSS SECTION SCHEMATIC DIAGRAM OF FILL AND DRAW PILOT UNIT

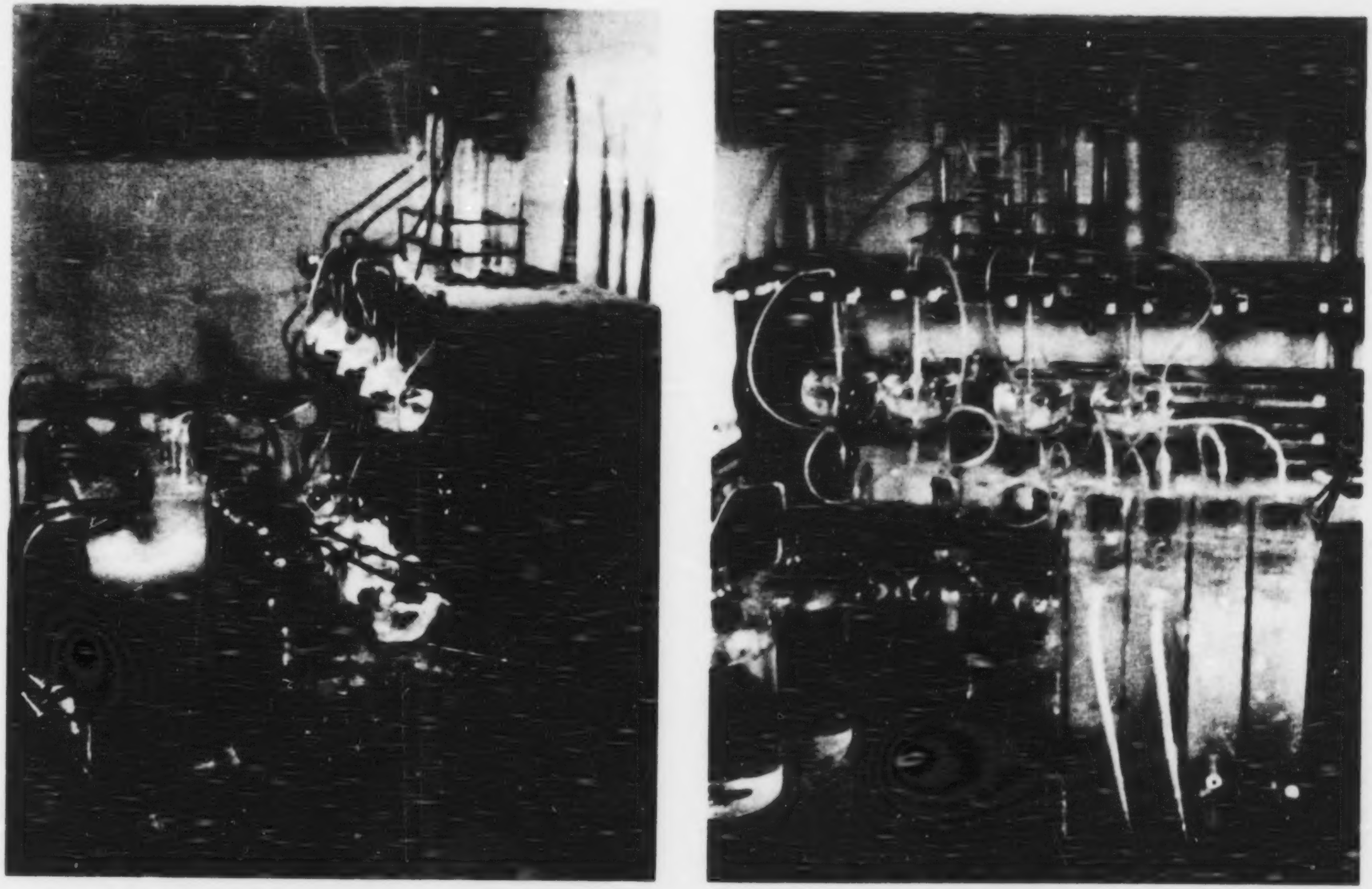


FIGURE 3 - MODEL ACTIVATED SLUDGE SYSTEM AND APPARATUS FOR CARBON BALANCE STUDIES

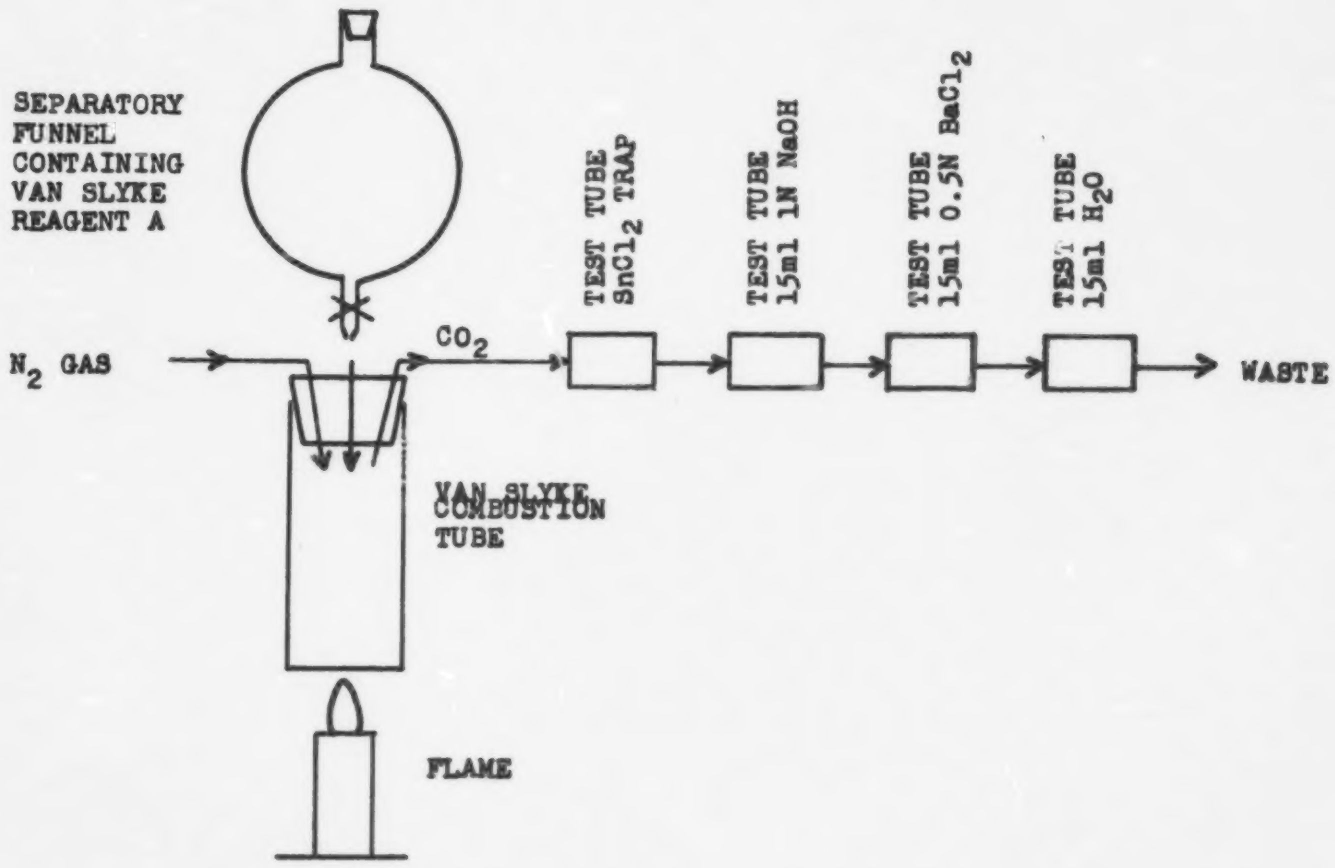

SEPARATORY FUNNEL CONTAINING VAN SLYKE REAGENT A
N_2 GAS
CO_2
TEST TUBE $SnCl_2$ TRAP
TEST TUBE 15ml 1N NaOH
TEST TUBE 15ml 0.5N $BaCl_2$
TEST TUBE 15ml H_2O
WASTE
VAN SLYKE COMBUSTION TUBE
FLAME

FIGURE 4 - SCHEMATIC DIAGRAM OF COMBUSTION APPARATUS

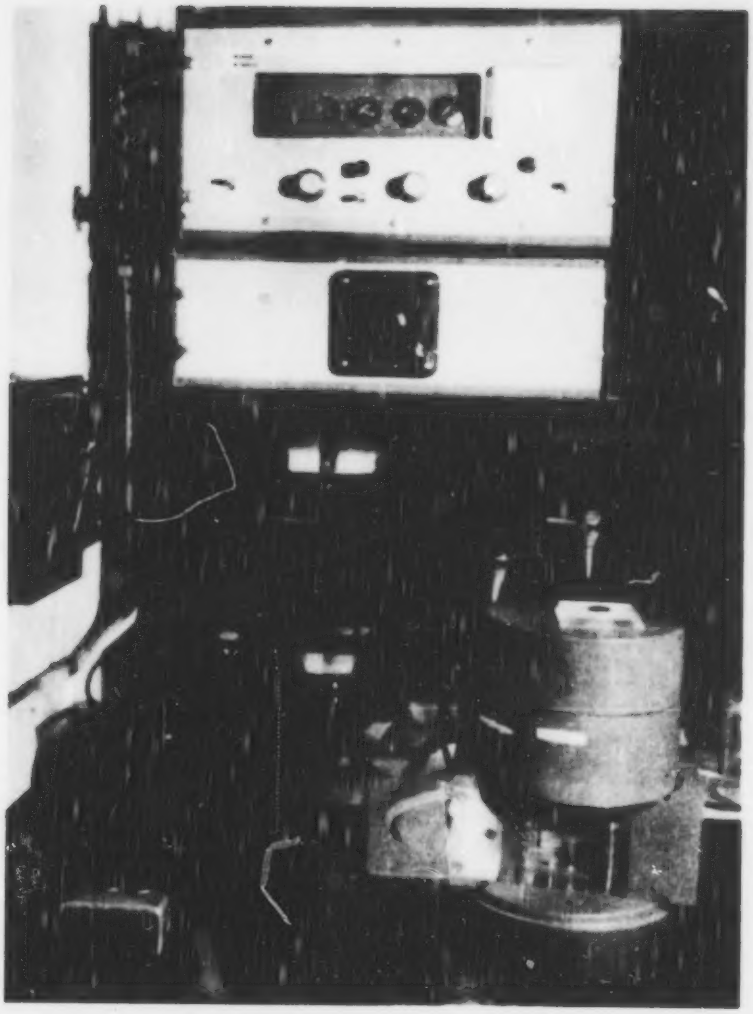


FIGURE 5 - BAIRD-ATOMIC RADIO-ASSAY EQUIPMENT

respiration, so that eventually the autolysis of the sludge approaches the magnitude of the sludge synthesized. When the maximum number of organisms which can develop and sustain themselves on the given amount of food are developed, the organisms operate in the stationary growth phase wherein no further accumulation of active sludge mass is possible. Considerable evidence has been reported which indicated that, under the specific conditions investigated, volatile sludge continues to accumulate under these equilibrium conditions. Unfortunately these reported incidences of accumulation at equilibrium have been related to a select group of substrates, determined over too short a period of time, or drawn on questionable data. It remains for this research to confirm this accumulation at equilibrium for substrates which are derivatives of the three major cell components. Little or no research has been reported concerning the composition of the volatile sludge solids which accumulate at equilibrium. It is possible, however, from a knowledge of the advances in bacteriology to theorize as to their composition. It is well known that most parts of bacterial cells are readily degradable by individual bacterial species or allied bacteria commonly developed from soil or sewage inoculum. The degradability of cell walls and slime, if developed, varies with the various species. In many cases it has been found that organisms are not able to autolysize their own slime. The destruction of such material is, therefore, left to other organisms which for various environmental conditions may not be present or predominate in activated sludge. As has been shown by a Literature Review, these difficult to degrade cell portions are composed of chains of various derivates depending upon the substrate used as a source of energy, as well as the organism and its growth phase. In the majority of cases reported, these materials were mostly, if not wholly, carbohydrate in nature. There were, however, some cases in which the slime layer was reported as entirely polypeptide in nature. Although this was the exception, a considerable number of researchers reported capsules had some nitrogenous derivatives. It is reasonable to suggest that in mixed culture, such as is obtained with a sewage inoculum, that any non-active, biologically inert organic material which accumulates in the system will, in fact, contain carbohydrates in some unknown ratio with compounds yielding nitrogenous derivates, and that the carbohydrate material will be in the majority by weight.

If indeed nitrogen is present in the material which accumulates at equilibrium, there would be considerable engineering significance in this finding. This information would contradict the conclusion of some researchers that long-term aeration systems can operate without the addition of nitrogen in excess of that lost out of the effluent. It

It may be necessary to give substantial nitrogen supplement when treating nitrogen deficient wastes regardless of the effluent conditions. Such a revelation may well prevent improper design of treatment systems and treatment of such industrial waste.

RESULTS

A. Long-Term Carbon Balance Studies

The long-term carbon balance studies were divided into two phases according to the type of activated sludge process studied. The first phase utilized the batch or fill and draw type activated sludge process which was biologically similar to the conventional activated sludge process. The second phase was conducted upon a continuous flow system biologically representing the complete mixing activated sludge process.

Uniform Concentration Activated Sludge

Carbon Balance: The carbon balance was conducted on sludge which was fed 1 gm. of sodium acetate as acetic acid per day. The data for the balance were taken for cultures up to the age of 20 weeks in the continuous flow basin. A summary of the long-term carbon balance is presented in Table I. It is worthy of note that the balances over the period of test had but three error of closings larger than 10 per cent and that well over 50 per cent of the carbon balances closed with less than 3 per cent error.

The first continuous flow basin activated sludge system was destroyed during the twelfth week in the basin by sodium hydroxide which siphoned into the aeration basin from the first trap. It was after this accident that 2 liter empty bottles were placed between the aeration chamber and the carbon dioxide collection traps.

The destroyed sludge was replaced by an eight week old sludge and the long-term carbon balance study resumed. By happenstance the sludge concentration in the second sludge very closely agreed with that measured for the first sludge at eight weeks. Solids were wasted in varying amounts after the thirteenth week of operation of the second activated sludge. The purpose for wasting was twofold. First, it was decided to maintain the solids level at around 7,000 mg per basin. Secondly, the effect of wasting in an equilibrium system, on the synthesis rate was to be observed.

TABLE I

SUMMARY OF CARBON BALANCE FOR CONTINUOUS FLOW BASIN FEED SODIUM ACETATE

Week in Basin	Carbon Removed mg	CO_2-C Recovered mg	Cell Growth-C mg	Effluent Cells-C mg	Cells Removed-C during Analysis mg	Balance mg-C	Closing Per Cent	Per Cent Oxidized
2	2665	2045	589	368	18	-346	13.0	77
3	2682	2274	-230	422	36	+180	6.7	85
4	2309	1918	100	370	28	-107	4.5	83
5	1124	993	90	14	40	-13	1.2	88
6	2696	2205	390	-	94	-	-	82
7	2452	2441	60	47	63	-159	6.5	99+
8	2663	1980	490	72	63	+58	2.2	74
9	2604	2121	290	7	123	+63	2.4	81
10	2496	2063	300	10	74	+49	2.0	83
11	2761	2200	180	7	76	+298	10.8	80
Weekly Average over 5-10 week period	2612	2163	285	59	82	+23	0.9	83
		System destroyed and replaced with 8 week old system.						
9	2638	2394	190	30	64	-40	1.5	91
10.5	3560	3880	-450	100	97	-67	1.9	109
13	5529	5166	120	240	106	-103	1.9	94
14	2255	2067	190	135	294	-431	1.9	92
16	5278	5001	-440	580	100	38	0.7	95
18	6453	5180	-490	147	1,910	-294	4.5	80
20	5800	5187	440	300	46	-83	1.4	89
Weekly Average over 9-20 week period	2700	2460	-41	128	218	-65	2.4	91

Although some of the difficulties did affect individual balance periods, the general trends of the data remain clear. The weekly average over the 5-10 week period of the first culture is given in Table I. Over this six week period the system was considered to be in equilibrium (see Figure 6). It was noted that the sludge continued to accumulate in the basin over the entire period and at an average rate of 285 mg carbon per week or about 9.0 mg sludge per gm of acetic acid. Also during this period the overall closing error was but 0.9 per cent. The entire period of balance on the second continuous flow basin sludge is averaged in Table I. Here there is an average decrease in the basin volatile solids as a result of heavy wasting.

Oxidation: The per cent of complete oxidation over balance periods is also given in Table I. These percentages represent the portion of the acetate carbon removed which was stabilized to carbon dioxide. The equilibrium system, as defined earlier, represents a condition wherein the active solids are a maximum for the given organic load. At equilibrium, the stabilization would approach 100 per cent if there were no solids wasted and no inert material accumulated in the system. If on the other hand, the inert solids continue to accumulate at equilibrium due to inert material synthesis, the per cent stabilization will approach a somewhat constant value provided the wasting is minimal. The latter case of inert accumulation took place under the conditions of this study. The percentage equilibrium stabilization for the first continuous flow system activated sludge ranged in the eighties with an average over the period of equilibrium operation of 83 per cent complete oxidation. During the operation with the second activated sludge culture, the average stabilization was considerably higher at 91 per cent and may have been still higher had sludge wasting not been practiced during a portion of its operation. For the purpose of this study, any system showing in excess of 80 per cent oxidation was considered to be in equilibrium operation.

Chemical Composition of Sludge: The chemical composition of the continuous flow system sludges was determined on six occasions during the course of the long-term carbon balance study. The resulting data was used to calculate empirical formula of the activated sludge solids and determine the per cent carbon content of the volatile solids presented in Table II. As mentioned previously, the per cent carbon was used to convert the volatile solid measurements to equivalent carbon for the carbon balance.

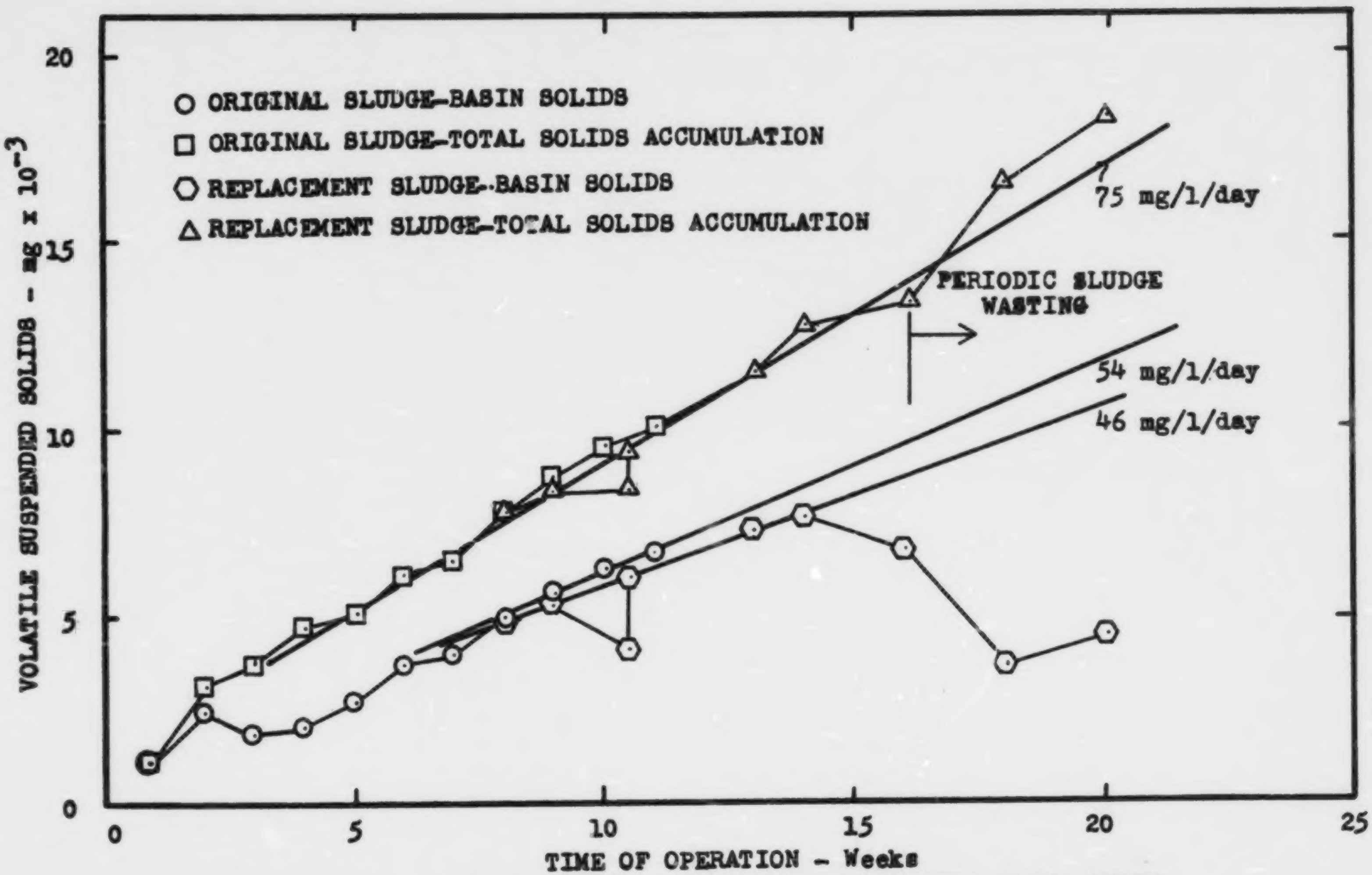


FIGURE 6 - ACCUMULATION OF SLUDGE IN CONTINUOUS FLOW SYSTEM

TABLE II

LONG-TERM CARBON BALANCE

EMPIRICAL FORMULAE OF CONTINUOUS FLOW SYSTEM SLUDGES

Week in Basin	Empirical Formula	Carbon, Per Cent of Volatile Solids	Organic Nitrogen, Per Cent of Volatile Solids
3	$C_5H_{9.9}O_{2.9}N_{1.0}$	0.454	10.4
7	$C_5H_{9.8}O_{2.5}N_{1.1}$	0.483	12.2
9	$C_5H_{10.4}O_{3.0}N_{1.0}$	0.460	10.2
9(1)	$C_5H_{10.3}O_{2.5}N_{1.1}$	0.480	11.7
13	$C_5H_{8.7}O_{2.6}N_{0.8}$	0.490	9.2
16	$C_5H_{9.6}O_{2.6}N_{1.0}$	0.478	10.7

(1) Original sludge destroyed on the 11th week and replaced with 8 week-old sludge.

Accumulation of Sludge: The accumulation of sludge in the whole system, as well as in the basin is graphically presented in Figure 6. The graph indicates that the system reached equilibrium between the fifth and sixth weeks at a solids level of about 4,000 mg/basin. This amounts to around 2800 mg/l MLVS. The dip in the basin solids curve from the second to the sixth week was the result of loss of poorly flocculated solids out the effluent. It may well be that, if it were not for this difficulty, equilibrium would have occurred after one or two weeks.

The total solids accumulation for both sludges give close agreement in the period of overlap. A straight line approximation of the sludge accumulation rate reveals that the system volatile solids continues to accumulate at a rate of 76 mg/l/day of valtile solids. The divergence from this mean rate of growth after the fourteenth week show a slight increase accumulation induced by sludge wasting.

The plot of the volatile solids in the basin (Figure 6) shows that the second culture was about one-half week behind the development of the first culture but their general pattern of growth were similar. The rate of sludge accumulation in the first system was 54 mg/l/day of volatile solids compared to 46 mg/l/day of volatile solids for the second culture. Although this difference is only 15 per cent, it was considered to be of some significance since these were average accumulations and therefore more precise than the individual solids observations which may be in error as much as 10 per cent. No attempt was made, however, to interpret this difference.

The accumulation data was of little significance after the fourteenth week of operation of the second activated sludge system due to the large amount of sludge wasting. This was especially true of the volatile solids content in the basin where the solids level dropped to about half its former value by the eighteenth week. At the 14 week mark, about 0.6 per cent of the MLVS was being wasted each day.

Conventional Activated Sludge

Carbon Balance: The summary of the carbon balance for this system operating over a period of 25 weeks is given in Table III.

It was noteworthy that the average sludge accumulation in the basins over the period of equilibrium operation (5-13 weeks) was substantially the same as obtained for the continuous flow system. The sludge accumulated at an average rate of 282 mg as carbon per week compared to 285 mg C/week for the continuous flow system. All but four of the balances had closing errors of less than 10 per cent. The average closing over the 5-13 weeks equilibrium period was only 2.2 per cent.

Oxidation: The per cent of complete oxidation over balance periods is given in Table III. These percentages represent the portion of the acetate which was stabilized to carbon dioxide. The average per cent stabilization during equilibrium (5-13) weeks was 84 per cent which agrees with the value found for the continuous flow system. Once wasting of sludge began the per cent stabilization again decreased to the seventies. Having a per cent stabilization in this range, indicates a system in the declining growth phase when operating at the same loading factor (1 gm C.O.D. per day per 1.5 liters of solution.)

TABLE III

SUMMARY OF CARBON BALANCE FOR FILL AND DRAW BASIN FEED SODIUM ACETATE

Week in Basin	Carbon Removed mg	CO_2-C Recovered mg	Cell Growth-C mg	Effluent Cells-C mg	Cells Removed-C during Analysis mg	Balance mg-C	Closing Per Cent	Per Cent Oxidized
2	2200	1,383	972	67	10	-232	10.5	63
3	2758	1,947	950	87	38	-264	9.6	70
4	2720	2,120	730	27	210	-367	14.0	78
5	3092	2,009	870	225	81	-93	3.0	65
6	2717	2,095	350	40	156	76	2.8	77
7	2546	2,171	230	85	232	-172	6.7	85
8	2695	2,208	260	25	114	88	3.3	82
9	2689	2,176	570	24	190	-271	10.1	81
10	2686	2,304	230	28	122	2	0.1	86
11	2706	2,420	250	25	140	-129	4.8	89
12	2748	2,335	350	13	205	-155	5.6	85
13	2658	2,300	0	18	147	193	7.3	86
14	2286	1,963	300	25	148	-150	6.6	86
16[1]	5605	4,221	900	315	240	-71	1.3	75
18	5690	4,207	650	510	200	123	2.2	74
19	2365	1,832	-650	325	825	33	1.4	77
21	5900	4,505	1400	342	330	-677	11.5	76
23	5485	3,886	360	438	890	-89	1.6	71
25	5800	4,055	100	320	1,370	-45	0.8	70
Weekly Average over 5-13 week period	2637	2,219	282	31	162	-57	2.2	84

1. Periodic sludge wasting occurred beyond the 16th week.

Chemical Composition of Sludge: The chemical composition of the fill and draw system activated sludge was determined on six occasions during the course of the long-term carbon balance study. Empirical formulae of the activated sludge solids were calculated from this data and reported in Table IV together with the per cent carbon content of the volatile solids.

The nitrogen content of the sludge decreased with time of operation of the fill and draw system. These data would then indicate that a non-nitrogen organic material was accumulating in the system. The nitrogen content of the sludge, however, dropped only 2.5 per cent, from 12 to 9.5 per cent, over the entire 25 weeks. Considering the reproducibility of the microchemical analysis, this small change in nitrogen content of the sludge cannot be considered conclusive evidence of polysaccharide build up in the system.

TABLE IV

LONG-TERM CARBON BALANCE

EMPIRICAL FORMULAE OF FILL AND DRAW SYSTEM SLUDGES

Week in Basin	Empirical Formula	Carbon Percent of Volatile Solids	Organic Nitrogen Percent of Volatile Solids
3	$C_5H_{9.7}O_{2.7}N_{1.1}$	0.470	12.2
7	$C_5H_{9.0}O_{2.3}N_{1.0}$	0.504	11.1
9	$C_5H_{10.7}O_{3.1}N_{1.0}$	0.540	10.6
12	$C_5H_{9.9}O_{3.0}N_{1.0}$	0.455	11.0
15	$C_5H_{10.9}O_{3.1}N_{1.0}$	0.449	10.3
18	$C_5H_{8.8}O_{2.6}N_{0.9}$	0.486	10.0
22	$C_5H_{9.0}O_{2.7}N_{0.8}$	0.481	9.3

Accumulation of Sludge: The accumulation of sludge in the system, as well as that accumulated in the basin, is graphically presented in Figure 7. The accumulation in the basin and that in the whole system (including wasting) diverged at a rather constant rate until the wasting of sludge began.

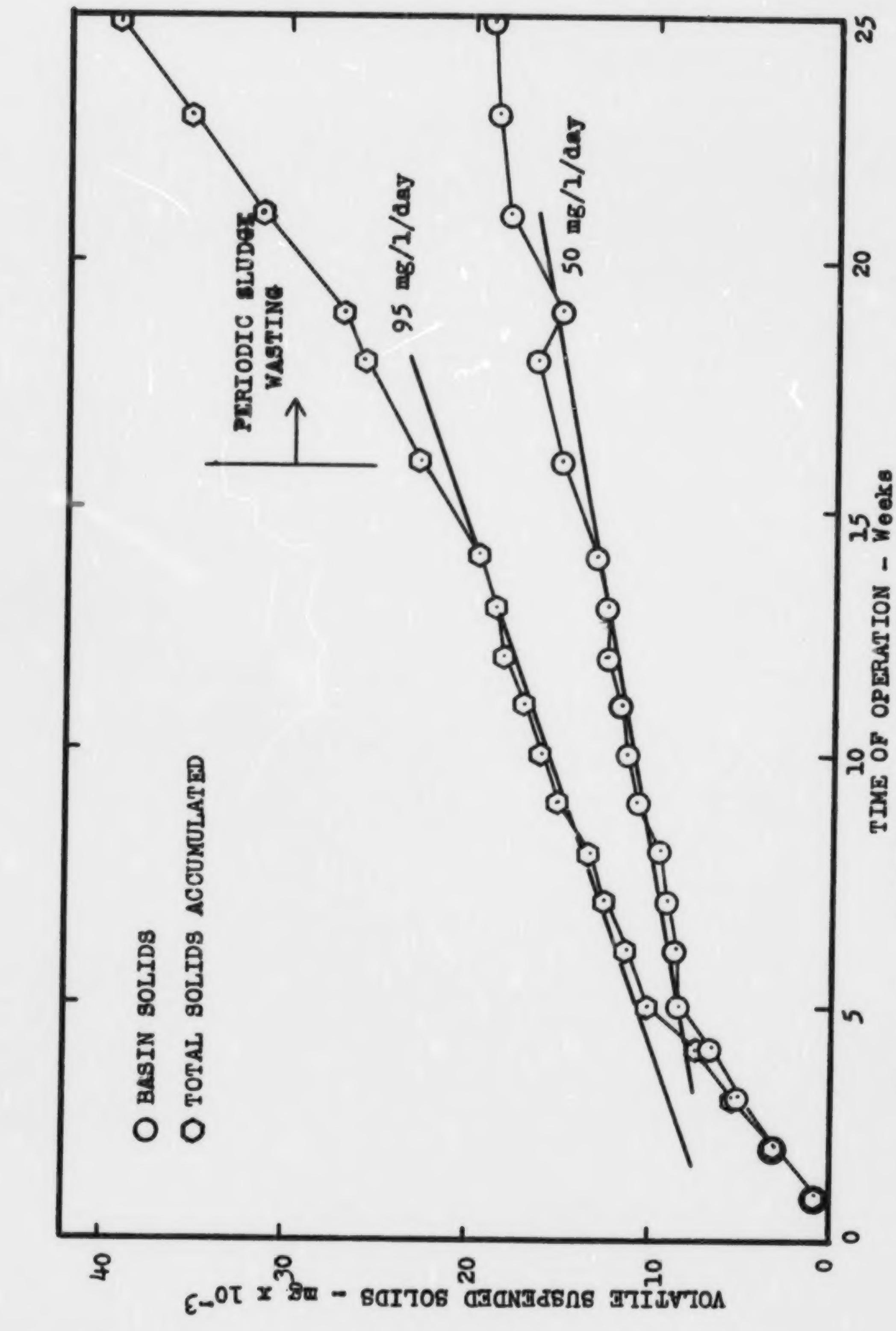


Figure 7 - ACCUMULATION OF SLUDGE IN FILL AND DRAW SYSTEM

The divergence was also shown by the data in the summary of the carbon balance Table III wherein the cells in the effluent together with those removed for analyses remain essentially constant up to the fourteenth week. As was expected the total solids further increased their rate of accumulation over that of the basin solids once wasting started.

The fill and draw system required five weeks to reach an equilibrium as indicated by the sharp drop in the rate of accumulation at that time. During equilibrium the volatile solids in the reactor basin increased at a rate of approximately 50 mg/l/day. The total production of sludge volatile solids was about 95 mg/l/day. At the 14 week mark about 0.67 per cent of the MLVS was wasted each day.

Summary of Long-Term Carbon Balance Results

In both systems after the fifth week of operation the rate of volatile solids accumulation decreased sharply. This break was taken as the start of equilibrium operation. The rate of increase of volatile solids in the reactor basins during equilibrium was essentially the same for both the continuously fed and the batch fed systems. The total production of cells during equilibrium was greater in the fill and draw system, however, than in the continuous flow system. This may indicate that the synthesis at equilibrium is also indipendent of the sludge wasted or lost in the effluent as long as that quantity lost is not so great as to upset the equilibrium condition of the reactor basin. The results of the carbon balance study are summarized in Table V.

TABLE V

SUMMARY OF SLUDGE ACCUMULATION DURING LONG-TERM STUDIES

	Uniform Concentration Systems		Conventional Systems
	First	Second	
Initial Volatile Solids at Equilibrium mg/l	2,800		5,000
Volatile Solids Accumulation at Equilibrium mg/l/day	54	46	50
Total Volatile Solids Production mg/l/day	75	75	95
Stabilization of Substrate at Equilibrium Percentage	83	91	84

Although there was a gradual decrease in the percentage of nitrogen in the volatile solids of both systems during equilibrium, the absolute amount of nitrogenous material continued to increase. These relationships are shown in Figure 8 wherein the volatile solids and the nitrogen content of both sludges are plotted over the period of study. As can be seen from this figure, after equilibrium the nitrogen content of the volatile solid fell below the 11.6 per cent value given for the average viable cells. The nitrogen did, however, continue to increase at a rate ranging from 8.2 to 9.0 per cent of the accumulated volatile solids.

B. Acetate-1-C^{14} Systems

This section, as well as the remaining three sections of results, is classified in accordance with the radioactive substrate used in the investigation. The purpose of these sections was to investigate the synthesis and autolysis rates of sludges components under equilibrium conditions for different substrates. It was felt that such data would show the influence of substrate on sludge accumulation and also determine the general component or components of which the inert volatile material was composed. As explained previously, in each section, duplicate continuous flow and fill and draw units were investigated. In all, sixteen systems were studied. For convenience, the systems are herein referred to by system or basin number in accordance with the following designations:

Systems	Radioactive Substrate	System Type
1-2	Acetate-1-C^{14}	Continuous flow*
3-4	Acetate-1-C^{14}	Fill and draw**
5-6	Acetate-2-C^{14}	Continuous flow*
7-8	Acetate-2-C^{14}	Fill and draw**
9-10	Glucose-u-C^{14}	Continuous flow*
11-12	Glucose-u-C^{14}	Fill and draw**
13-14	Glycine-1-C^{14}	Continuous flow*
15-16	Glycine-1-C^{14}	Fill and draw**

* Uniform concentration activated sludge
** Conventional activated sludge

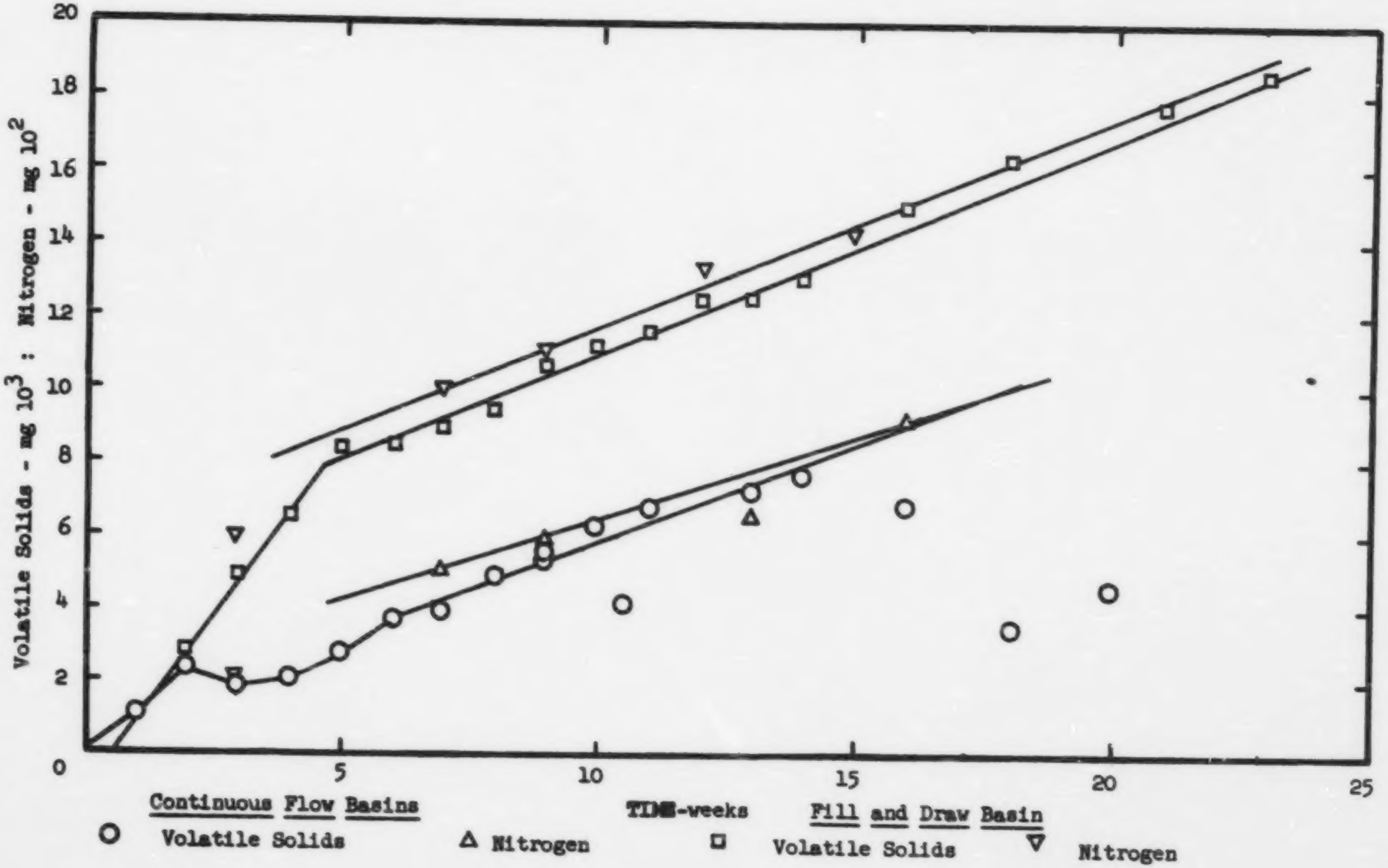


FIGURE 8 - NITROGEN AND VOLATILE SOLIDS ACCUMULATION DURING THE LONG-TERM CARBON BALANCE

Each section was composed of three study phases. These phases were, in the order of their occurrence, carbon balance, synthesis, and autolysis. The third phase of the carboxyl-C^{14} acetate investigation differed from that of the other section in that, rather than chemically measuring the effect of degradation on the sludge components, the change in the specific activity with time of degradation was measured. This study was made to observe the heterogeneous nature of the equilibrium sludge volatile solids due to build-up in the system of material, other than the active cells, which was not easily metabolized by the active cells. This excess material is herein referred to as inert solids.

Carbon Balance: The carbon balances were conducted from conception through the fifteenth week of growth. The summary of the carbon balances for the continuous flow systems #1 and #2 is given in Table VI. A similar summary of carbon balances for the fill and draw systems #3 and #4 is given in Table VII. Over the period of study the carbon balances for all four basins were continually within a closing error of 10 per cent except for the last balance on both continuous flow systems which gave a 17 per cent error of closing for basin #1 and 12.8 per cent for basin #2. The cause of these high values was not readily discernible. Apparently all systems reached equilibrium operation between the fifth and seventh week of growth. The flow through units continued equilibrium operation through the fifteenth week of this initial phase of the study. Wasting was practiced in the fill and draw basins after the tenth week of operation. Excess wasting resulted in high synthesis and less than equilibrium stabilization on the thirteenth week balance and to a lesser extent on the fifteenth week balance. Since the synthesis phase of this study was not started until after the sixteenth week of operation, there being little wasting between the conclusion of the carbon balance and start of the synthesis phase of study, equilibrium conditions were assumed to exist at that time.

The chemical composition of the activated sludges developed in these systems was measured on the third and thirteenth week of growth. It was noted that the nitrogen content of all sludges decreased over the ten week period. The fill and draw systems sludges showed the largest reduction in nitrogen which would indicate a proportionately larger nitrogen free volatile solids accumulation in those basins.

TABLE VI

SUMMARY OF CARBON BALANCE FOR CONTINUOUS FLOW BASINS - CARBOXYL C^{14} ACETATE

1	2	3	4	5	6	7	8	9
Week in Basin	Carbon Removed mg	CO_2-C Recovered mg	Cell Growth-C mg	Effluent Cells-C mg	Cells-C Removed during Analysis mg	Balance mg-C	Closing[1] Per Cent	Per Cent[2] Oxidized
System 1 - Continuous Flow								
3	6,837	5,250	994	844	0	-250	3.5	76
5	5,170	5,410	726	184	60	--	--	80
7	5,790	5,400	-20	340	47	30	0.5	93
10	7,600	6,670	870	164	46	-150	1.8	88
13	8,100	6,814	410	466	136	270	3.4	84
15	4,650	4,484	-940	36	397	850	17.0	96
System 2 - Continuous Flos								
3	6,895	5,460	948	490	0	-100	1.4	80
5	5,245	4,898	1092	496	57	--	--	69
7	5,630	5,815	-390	263	56	-120	2.0	103
10	7,720	6,600	790	253	32	50	0.6	85
13	8,100	6,740	500	282	128	450	5.4	84
15	4,550	3,790	-370	183	332	610	12.8	83

1. Calculated based on total substrate added.

2. Balances on 5th week were in error due to poor prepurification of air. Therefore, per cent oxidation was calculated from total cell growth.

TABLE VII

SUMMARY OF CARBON BALANCE FOR FILL AND DRAW BASINS - CARBOXYL C^{14} ACETATE

1	2	3	4	5	6	7	8	9
Week in Basin	Carbon Removed mg	CO_2-C Recovered mg	Cell Growth-C mg	Effluent Cells-C mg	Cells-C Removed during Analysis mg	Balance mg-C	Closing[1] Per Cent	Per Cent[2] Oxidized
System 3 - Fill and Draw								
3	6,834	5,670	886	381	0	-100	1.4	82.5
5	5,195	5,021	984	290	53	--	-	74
7	5,914	4,600	1410	71	50	-210	3.5	78
10	7,929	5,330	1770	210	43	570	7.1	74
13	8,110	5,930	310	390	1480	0	0	73
15	4,600	3,690	-60	760	673	-460	9.5	80
System 4 - Fill and Draw								
3	6,800	5,580	1125	243	0	-150	2.1	82.0
5	5,310	4,834	1015	233	67	--	-	74
7	6,190	5,735	450	49	57	-95	1.5	92
10	8,313	6,100	1110	390	35	680	8.1	73
13	8.150	5,710	000	690	1210	540	6.6	71
15	4,600	3,525	-270	1060	615	-330	6.9	77

1. Calculated based on total substrate added.

2. Balances on 5th week were in error due to poor prepurification of air. Therefore, per cent oxidation was calculated from total growth.

Synthesis: The systems were made radioactive by the introduction of sodium acetate-1-C^{14} labeled substrate diluted with carrier sodium substrate. In this first study the activity of protein, fat, and carbohydrate fraction of the sludge was measured for synthesis during the initial four hours after radioisotope ingestion. The assays were continued, at frequent intervals, through the twenty first day to determine autolysis characteristics.

The assimilation in the continuous flow basins (Figures 9-10) during the first two hours of feeding was taken as a per cent of that portion of the substrate fed up to the time of sampling. The resulting approximately level per cent assimilation over the entire four hours of synthesis observations confirmed the previously presented hypothesis that the rate of synthesis and the total synthesis in a continuous flow system were identical. The rise in the protein curve will be discussed later. It is also apparent that the substrate was utilized continuously as applied. This was not the case in the batch feed system (Figures 11-12) where it took about two hours for the microorganisms in these sludges to utilize the daily supply of nutrient. The total assimilation did, however, level off once the substrate was exhausted.

The total assimilation of sodium acetate when taken as the sum of the cellular components assimilation was, in all systems, greater than the per cent synthesis measured in Long-Term Carbon Balance Section for activated sludge systems operating at equilibrium. Although these results seemed at first thought to contradict known biological theory and previously observed data, it was actually to be expected and can easily be explained. The per cent synthesis, as obtained from consideration of the stabilization during equilibrium operation of the systems studied in the previous section, was the average accumulation of sludge which took into account the assimilation or true synthesis of cells and the destruction of a portion of the sludge mass by autolysis over the entire time of aeration. The assimilation data on the other hand included true synthesis with only a slight amount of degradation occurring over the two hour period of substrate utilization.

The composition of the synthesized cells based on the per cent assimilation as computed above is presented in the right hand scale of Figures 9-12. The per cent of the components in the synthesized cells was calculated on the basis that the three components composed the total cell synthesis. The composition of the newly synthesized cells is presented below:

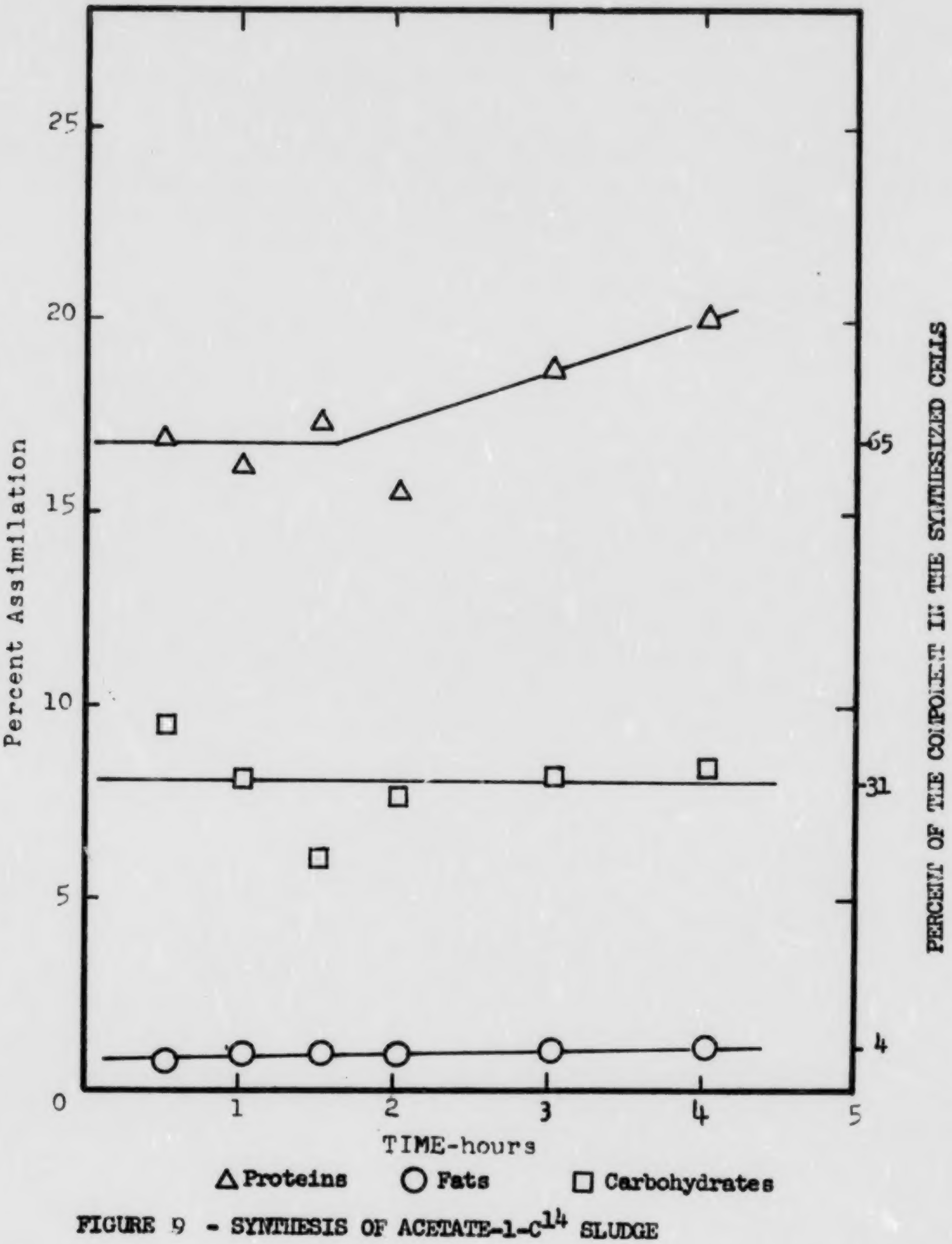


FIGURE 9 - SYNTHESIS OF ACETATE-1-C^{14} SLUDGE

BASIN #1

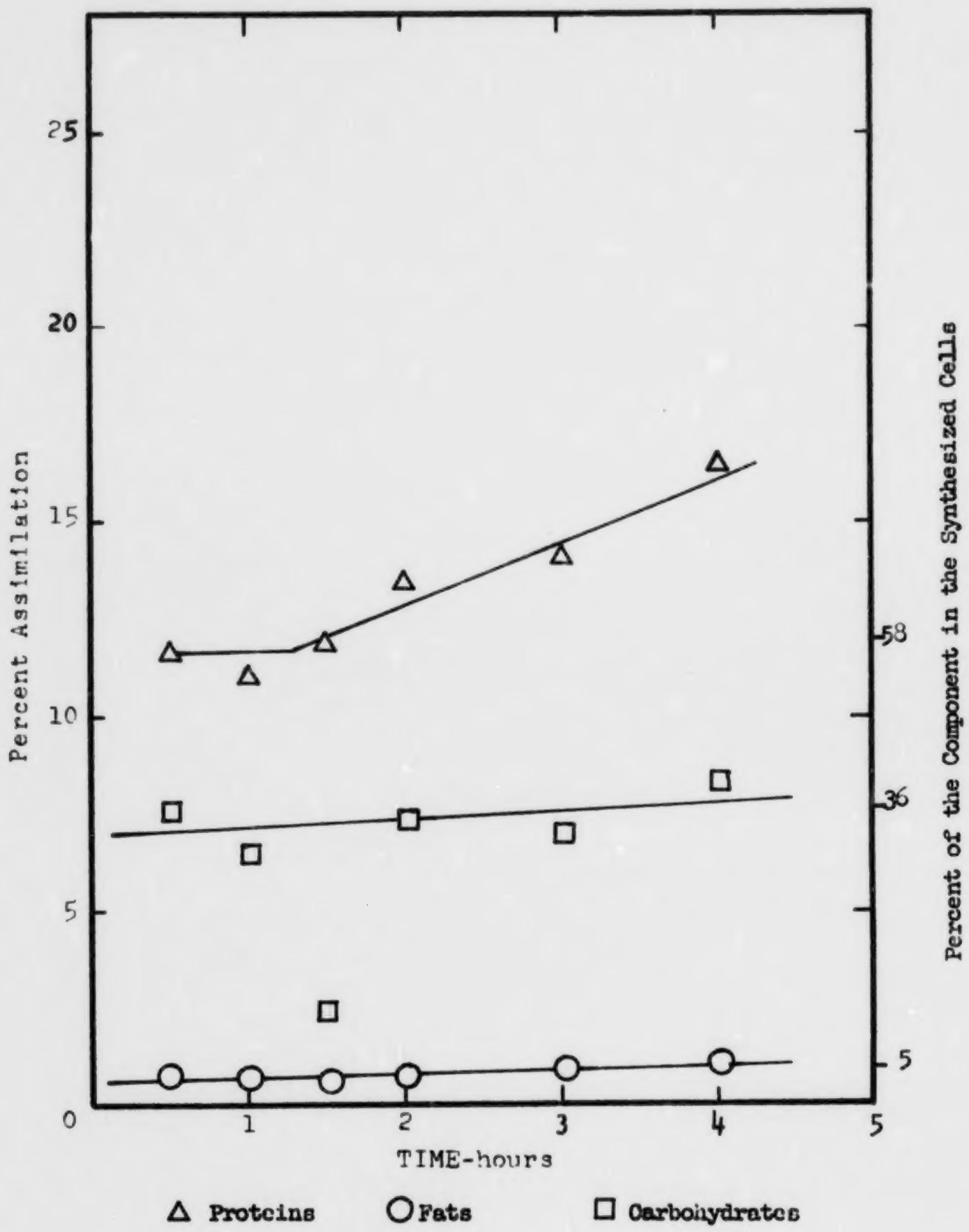


FIGURE 10 - SYNTHESIS OF ACETATE-1-C^{14} SLUDGE

BASIN #2

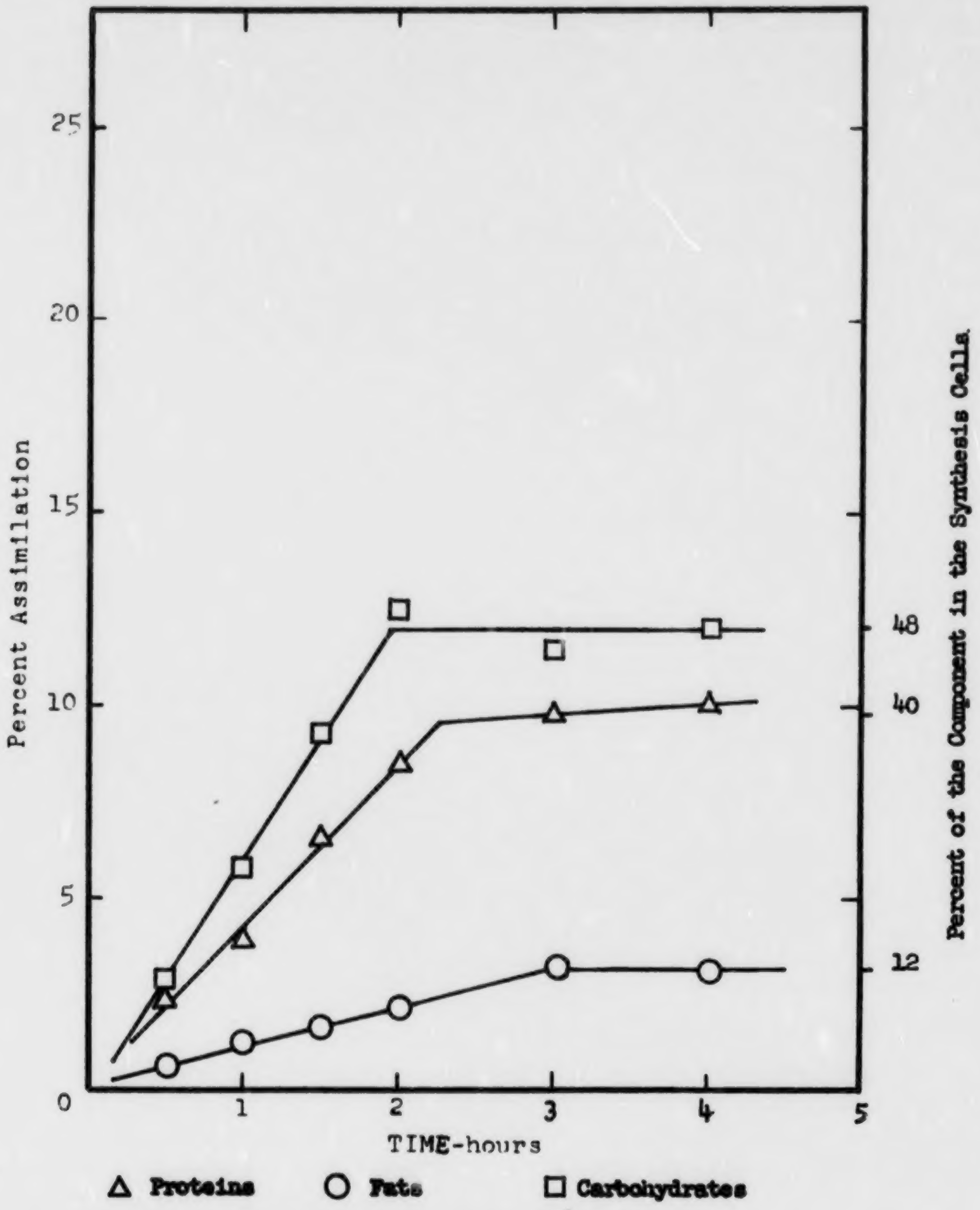


FIGURE 11 - SYNTHESIS OF ACETATE-1-C^{14} SLUDGE

BASIN #3

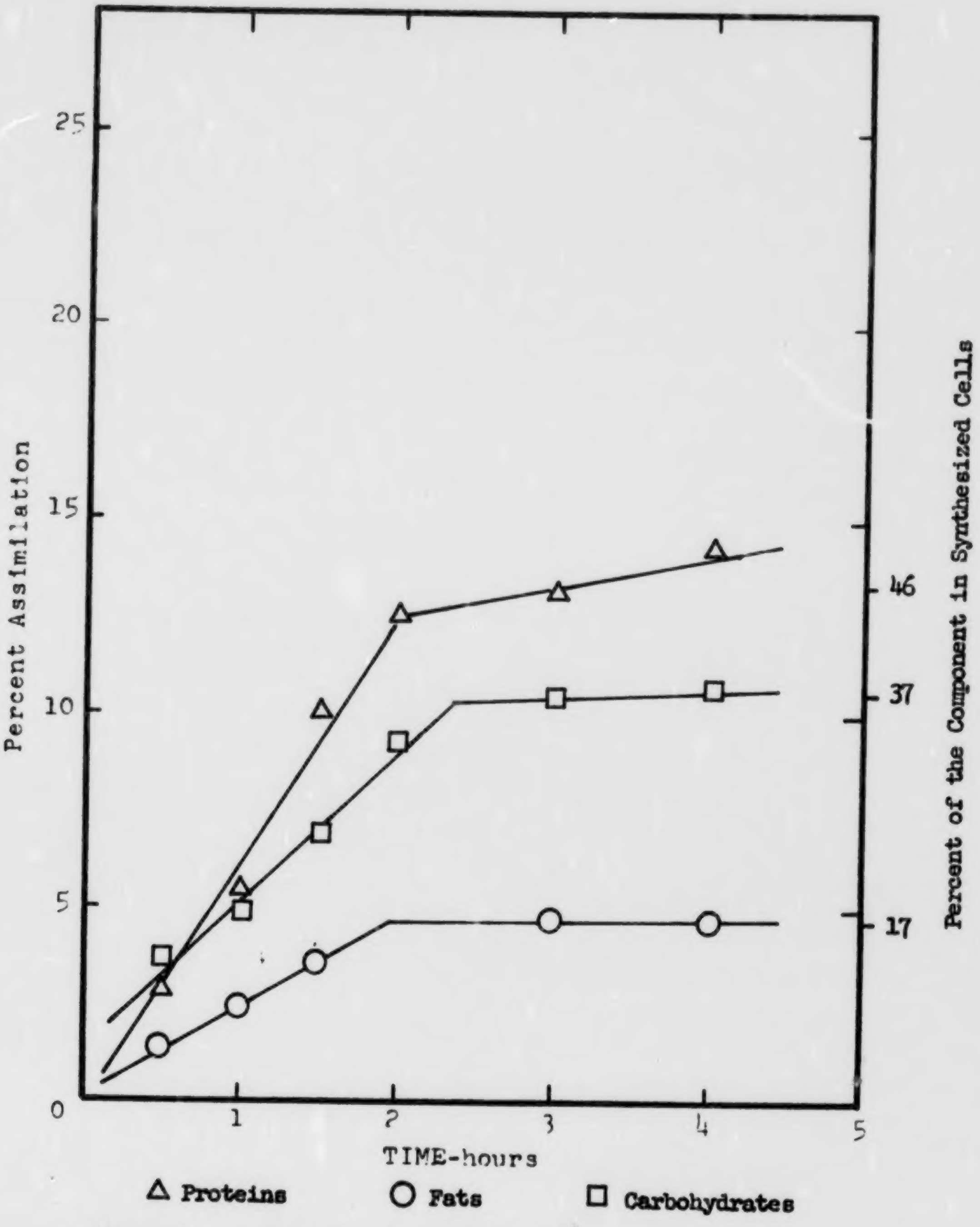


FIGURE 12 - SYNTHESIS OF ACETATE-1-C^{14} SLUDGE

BASIN #4

PER CENT COMPOSITION OF SLUDGES SYNTHESIZED FROM ACETATE-1-C^{14}

Component	Basin #1	#2	#3	#4
Protein	65	58	40	46
Carbohydrate	31	36	48	37
Fat	4	5	12	17

The values for the continuous flow systems sludge composition are reasonable when compared to the values given in the literature. The batch basin values are in obvious disagreement with what would be expected. The fat synthesis in the batch fed systems is felt to be higher than in the flow through systems because the active cells are exposed to an abundance of food which allows them to develop the observed high fat concentration of new cells. This excess fat portion is then used for endogenous respiration over the ensuing nonfed portion of the feed cycle.

The fact that the calculated carbohydrate synthesis is a considerable higher portion of the total synthesized active cell than that expected for active cells is a good indication of the existence of large quantities of external polysaccharides in the batch system. The difficult to degrade carbohydrate portion of the inert material was not completely reduced to sugars upon hydrolysis. When these isolated carbohydrate samples were purified through ionic resin, these nondegraded polysaccharides were removed because of their ionic properties. Consequently the radio assay sample didn't contain a representative sample of the carbohydrates as assumed in the calculations. From this discussion, it can be concluded that the assumption made in the calculation of the synthesis of the carbohydrate fraction, i.e., that the carbohydrate derivatives in the sample were identical to the carbohydrates calculated by difference, decreased in accuracy with increased polysaccharides in the sludge.

The synthesis of protein in the several acetate sludges is presented in Figure 13. The synthesis in the fill and draw basins show very good agreement in their

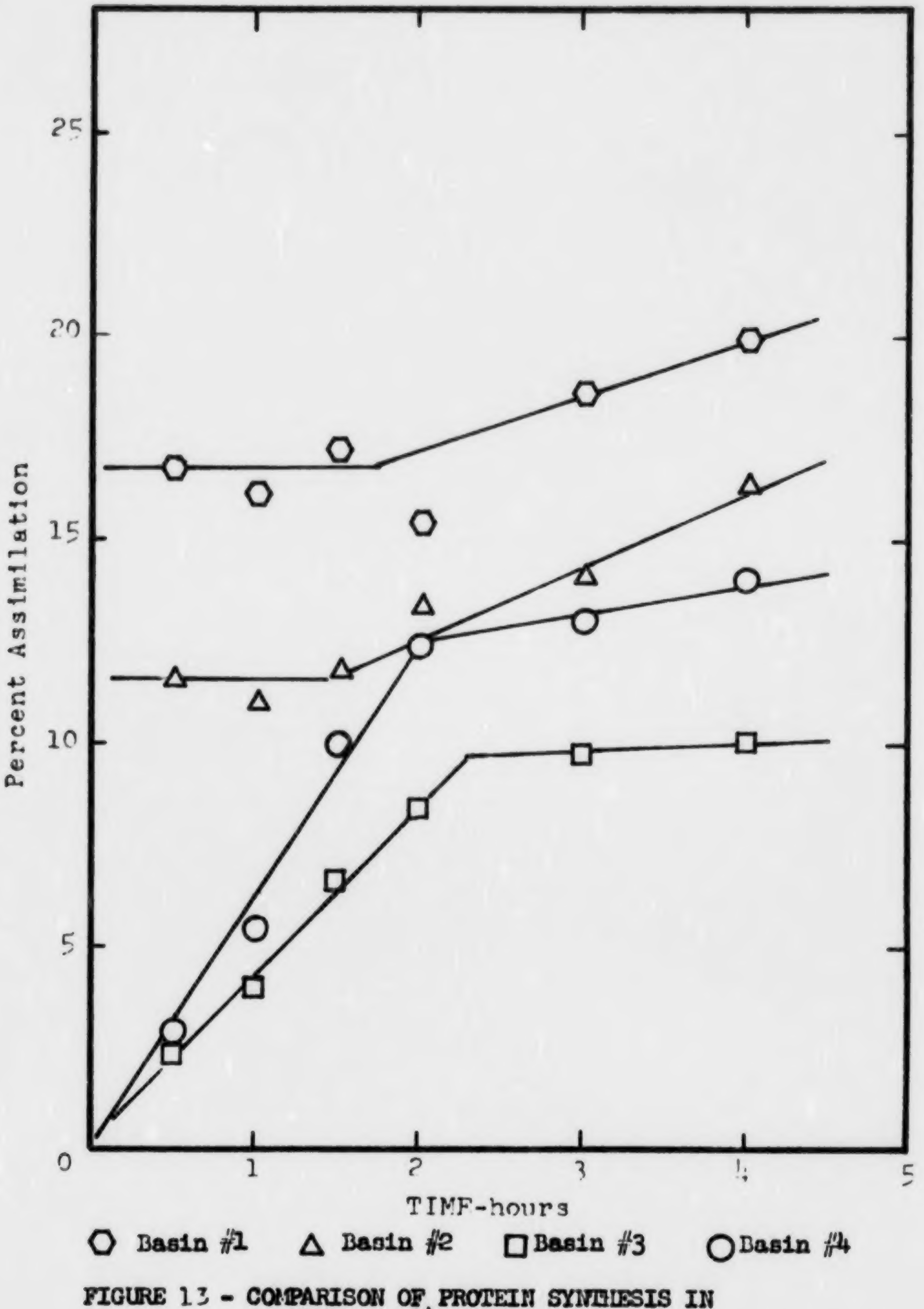


FIGURE 13 - COMPARISON OF PROTEIN SYNTHESIS IN ACETATE-1-C^{14} SYSTEMS

synthesis pattern. The general pattern of the synthesis in the continuous flow systems are similar with basin #2 having less protein than basin #1. The per cent assimilation of protein in all basins increased after the utilization of substrate. This apparent increase in sludge protein synthesis could have been the result of not supplying the usual amount of pH buffer to the systems with the inoculated radioactive substrate. The resulting higher pH value, caused the carbon dioxide, evolved in the energy reaction, to be absorbed into the solution where it was available for reincorporation into the various cell components by some form of CO_2 incorporation.

Autolysis: The specific activity of all components from all basins showed a decline over the three weeks of degradation. This decline is shown most clearly in Figure 14 wherein the per cent of radioactive feed remaining in sludge protein was presented for each basin. The specific activity continued to increase through the second day following the inoculation of the systems with radioactive acetate as a result of continued incorporation of labeled carbon dioxide. Between the second and third day, the specific activity reached a maximum in all basins. At these maxima, the increase in specific activity due to the carbon dioxide incorporation, probably resulted, for the most part, from the metabolic activities of autotrophic organisms after this long endogenous respiration period, was equalled by the decrease exponentially. After about a week the rate of decrease of the specific activity declined markedly. This change indicated either a mrked reduction in the activity of the sludge organisms or an adjustment to the more homogeneous degradation of the sludge by the remaining active organisms. The former seems most likely for the activity of the microorganisms decreases as the concentration of a required nutrient decreases. A concentration level is eventually reached at which the organisms die or enter a lag phase.

The reduction in specific activity for all sludge components over the 27 days of autolysis is presented in Table VIII.

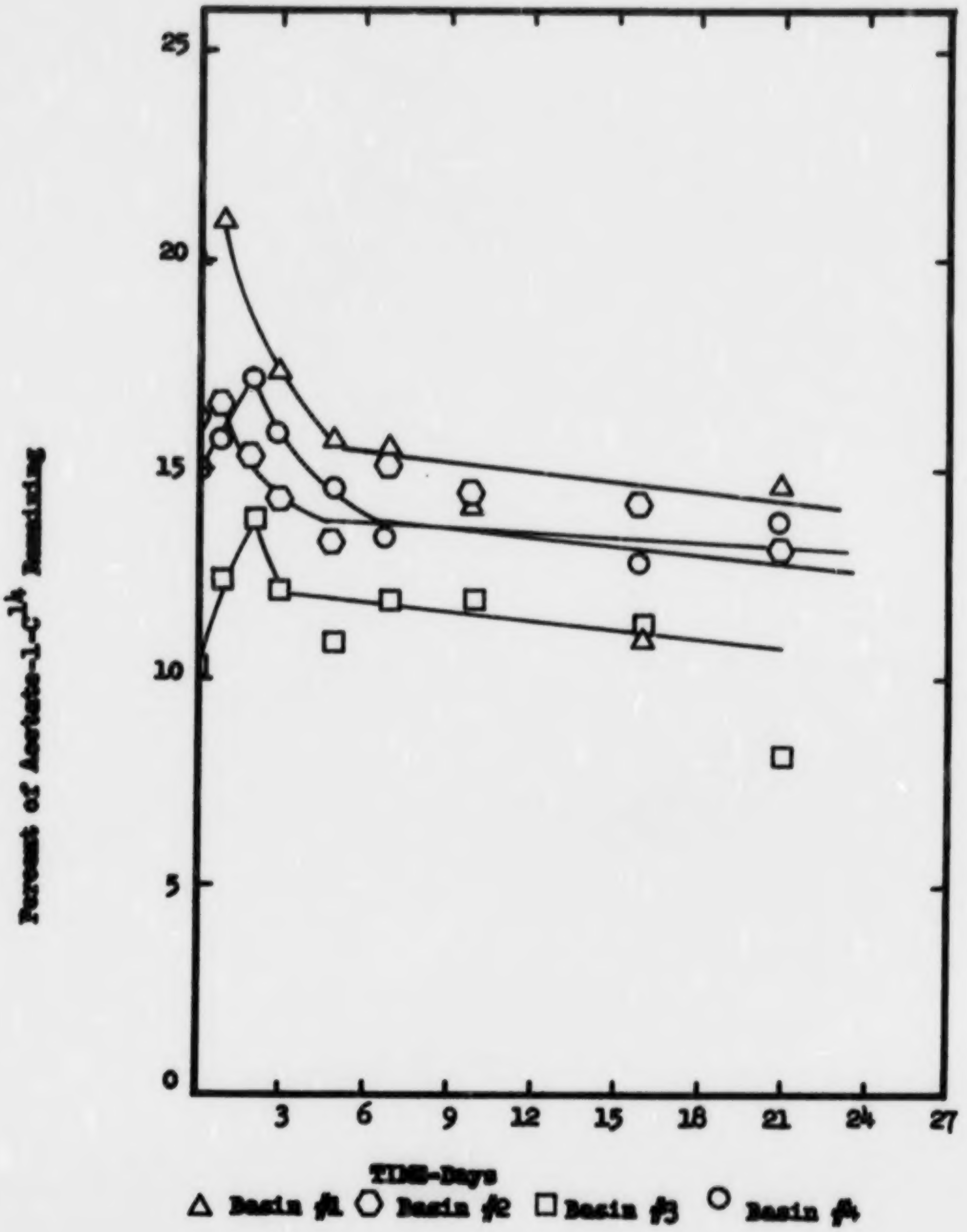


FIGURE 2A - PERCENT OF RADIOACTIVE FEED REMAINING IN SLUDGE PROTEIN DURING DEGRADATION

TABLE VIII

REDUCTION IN SPECIFIC ACTIVITY DURING AUTOLYSIS OF ACETATE -1-C-^{14} SLUDGES

	Percent Activity Remaining After 27 Days					
	Basin					
	Continuous Flow			Fill and Draw		
Component	#1	#2	Avg	#3	#4	Avg
Proteins	70	81	75½	58	67	62½
Fats	68	71	69½	57	74	65½
Carbohydrates	40	43	41½	27	33	30

All the component fractions for both the flow through and the batch systems showed a reduction in specific activity over the three week period of autolysis. It is noteworthy that the per cent specific activity remaining in the continuous flow systems average 10 percent higher for all components than that for the batch units. The lowest residual specific activity in all basins was that of the carbohydrate fraction which was about half the value for the other components for a given system.

As was noted earlier, not all the carbohydrate fraction was present in the radio assayed samples as a result of the ionic character of some of the hydrolysis products of the external polysaccharides. That portion of the inert material which did not enter the assayed sample would have further reduced the specific activity over the autolysis period. The inert carbohydrate fraction was then even greater than implied by Table VIII. Because of this inert material, the newly formed labeled cells were submitted to endogenous respiration and autolysis earlier than would have occurred if the whole system were degradable.

Summary: The systems were all operating at the equilibrium before the inoculation of the acetate-C^{14} substrate. The total volatile solids of the continuous flow systems at this time were about half those of the fill and draw system. Micro-analysis revealed that the nitrogen content of all sludges had measurably decreased from the third to thirteenth week of operation. The acetate-1-C^{14} was utilized by the activated sludge in the fill and draw systems in about two hours. The resulting assimilation was about 25 per cent in both systems with the major portion of new protoplasm being protein in nature. The continuous flow systems both utilized the substrate as it was fed. The fat synthesis was four or five times higher in the fill and draw systems than in the continuous fed systems. This fat accumulation was felt to be a temporary increase due to the high initial F:M. It evidently was utilized during the detention period for the analysis of the sludge indicated that the fats in the sludge mass were less for batch systems than for continuous flow systems.

There was an apparent continued assimilation of radioactive substrate in the protein fraction after the acetate had been metabolized. The assimilation was the result of carbon dioxide incorporation by both heterotrophic and autotrophic bacteria. Although the continuous flow systems appeared to have much higher rate of incorporation than the batch systems according to Figure 13, upon calculation it was shown that the batch units had a rate of incorporation about twice that for the continuous flow units. It was estimated that the heterotrophs incorporated about 0.5 mg c/hr which was a reasonable value when compared with the literature.

The autolysis data showed that over the period of investigation all components of all the sludges had a reduction in specific activity. The reduction was more pronounced in the fill and draw systems than in the continuous flow systems. The lowest residual specific activities occurred in the carbohydrate fractions which were about half the value for the other components.

C. Acetate-2-C^{14} Systems

Carbon Balance: For all the systems, collection of evolved carbon dioxide was started on the seventh week. The first balance was taken on the tenth week with the last balance being taken on the fourteenth week. The summary of the carbon balances for the continuous flow systems #5 and #6 (Table IX) shows that these systems were operating at equilibrium over the period during which carbon balance data were collected. The summary of the carbon balances for the fill and draw systems #7 and #8 is presented in Table X. The stabilization indicated by the tenth week carbon balance was below

TABLE IX

SUMMARY OF CARBON BALANCE FOR CONTINUOUS FLOW BASINS - METHYL C^{14} ACETATE

1 Week in Basin	2 Carbon Removed mg	3 CO_2-C Recovered mg	4 Cell Growth-C mg	5 Effluent Cells-C mg	6 Cells-C Removed during Analysis mg	7 Balance mg- C	8 Closing[1] Per Cent	9 Per Cent Oxidized
System 5 - Continuous Flow								
7								
10	9200	7510	1380	136	27	10	0.1	83
12	5290	4610	460	146	90	14	0.3	88
14	4050	3580	-370	63	150	500	12.3	88
System 6 - Continuous Flow								
7								
10	9150	7700	1210	111	29	-107	1.0	84
12	5080	4040	700	40	93	207	4.0	80
14	4050	3500	180	54	165	150	3.7	87

1. Calculated on basis of total substrate added.

TABLE X

SUMMARY OF CARBON BALANCE FOR FILL AND DRAW BASINS - METHYL C^{14} ACETATE

1 Week in Basin	2 Carbon Removed mg	3 CO_2-C Recovered mg	4 Cell Growth-C mg	5 Effluent Cells-C mg	6 Cells-C Removed during Analysis mg	7 Balance mg-C	8 Closing[1] Per Cent	9 Per Cent Oxidized
System 7 - Fill and Draw								
7								
10	9350	6270	2480	400	36	164	1.7	67
12	5340	3710	1500	220	145	-220	4.1	70
14	4127	2975	-160	136	960	216	5.0	72
System 8 - Fill and Draw								
7								
10	9350	6010	1690	1200	43	407	4.3	65
12	5285	3650	1280	590	135	-348	6.6	69
14	4257	2680	-110	560	875	252	6.0	63

---

1. Calculated on basis of total substrate added.

that for equilibrium operations in both systems #7 and #8. Non-equilibrium conditions existed in the basin despite the fact that the total volatile solids level was greater than that found in the long-term carbon balance study at initial equilibrium. This was probably due to the fact that the effluent for both systems was very turbid resulting in an unintentional sludge wasting. After the tenth week, periodic wasting was practiced resulting in stabilization of less than 72 per cent in the fill and draw systems during the remainder of the carbon balance phase of the study.

Synthesis: The systems were made radioactive by the introduction of sodium acetate-2-C_{14} labeled substrate diluted with carrier sodium acetate. These synthesis data for the components in each of the basins #5-#8 had been plotted in Figure 15 through 18. The synthesis curves were drawn through the experimental points by eye. The initial observations on the continuous fed systems were not considered to be as reliable as the other observations because there was considerably less C^{14} in the systems during these measurements due to the feeding procedure.

The right hand scale of the synthesis figures (Figures 15-18) indicates the apparent composition of the newly synthesized cells. These percentages were calculated on the assumption that the total synthesis was the summation of that of the three cell components, proteins, fats and carbohydrates. The composition of the newly synthesized cells thus calculated is shown below:

PER CENT COMPOSITION OF SLUDGES SYNTHESIZED

FROM ACETATE-2-C^{14}

	Basin			
Component	#5	#6	#7	#8
Protein	46	41	43	42
Carbohydrate	50	56	46	47
Fat	4	3	11	11

As was expected the horizontal nature of the curves indicated that the sludge was metabolized as it was fed in the continuous flow systems. Consequently, the calculations

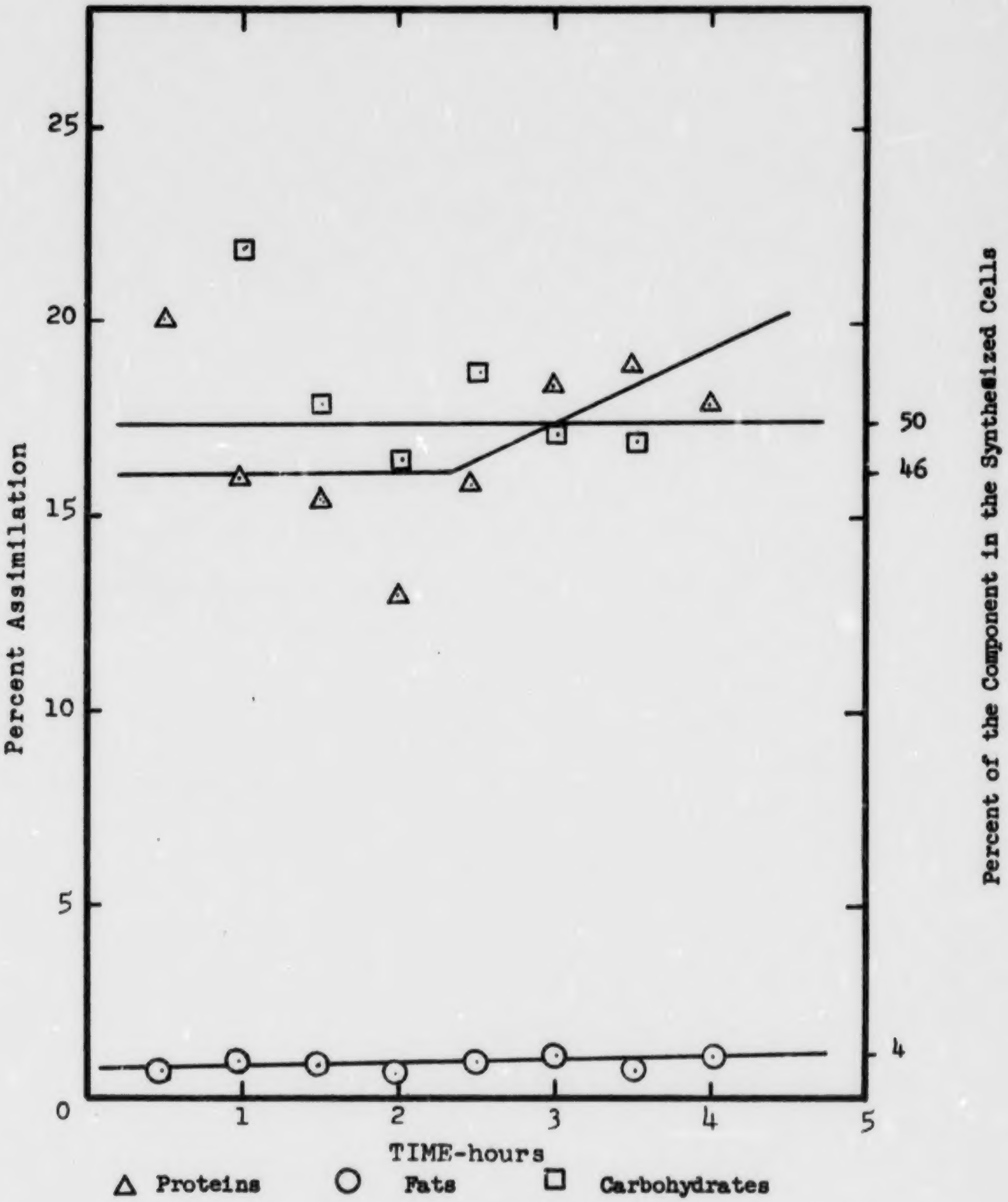


FIGURE 15 - SYNTHESIS OF ACETATE -2-C^{14} SLUDGE

BASIN #5

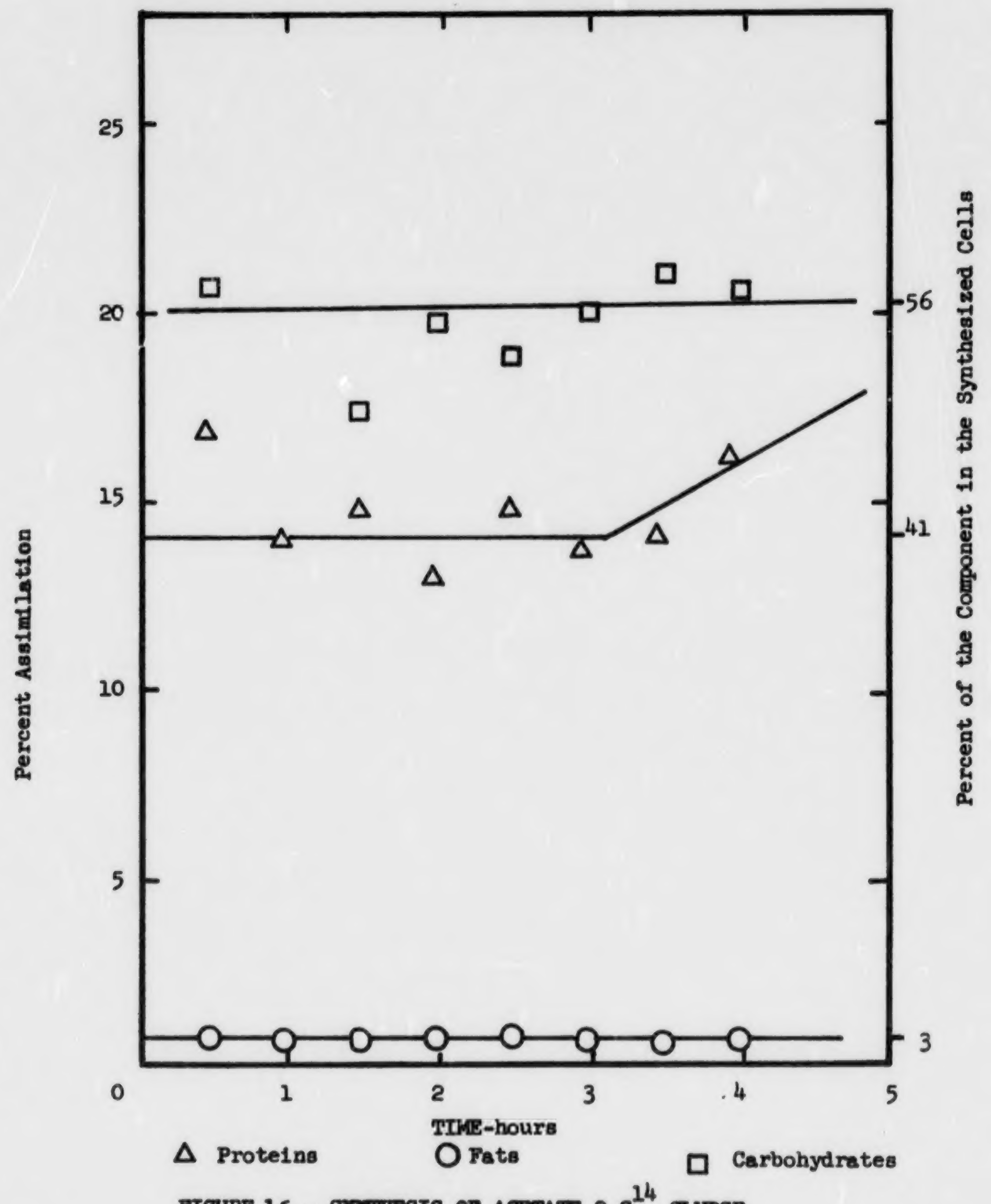
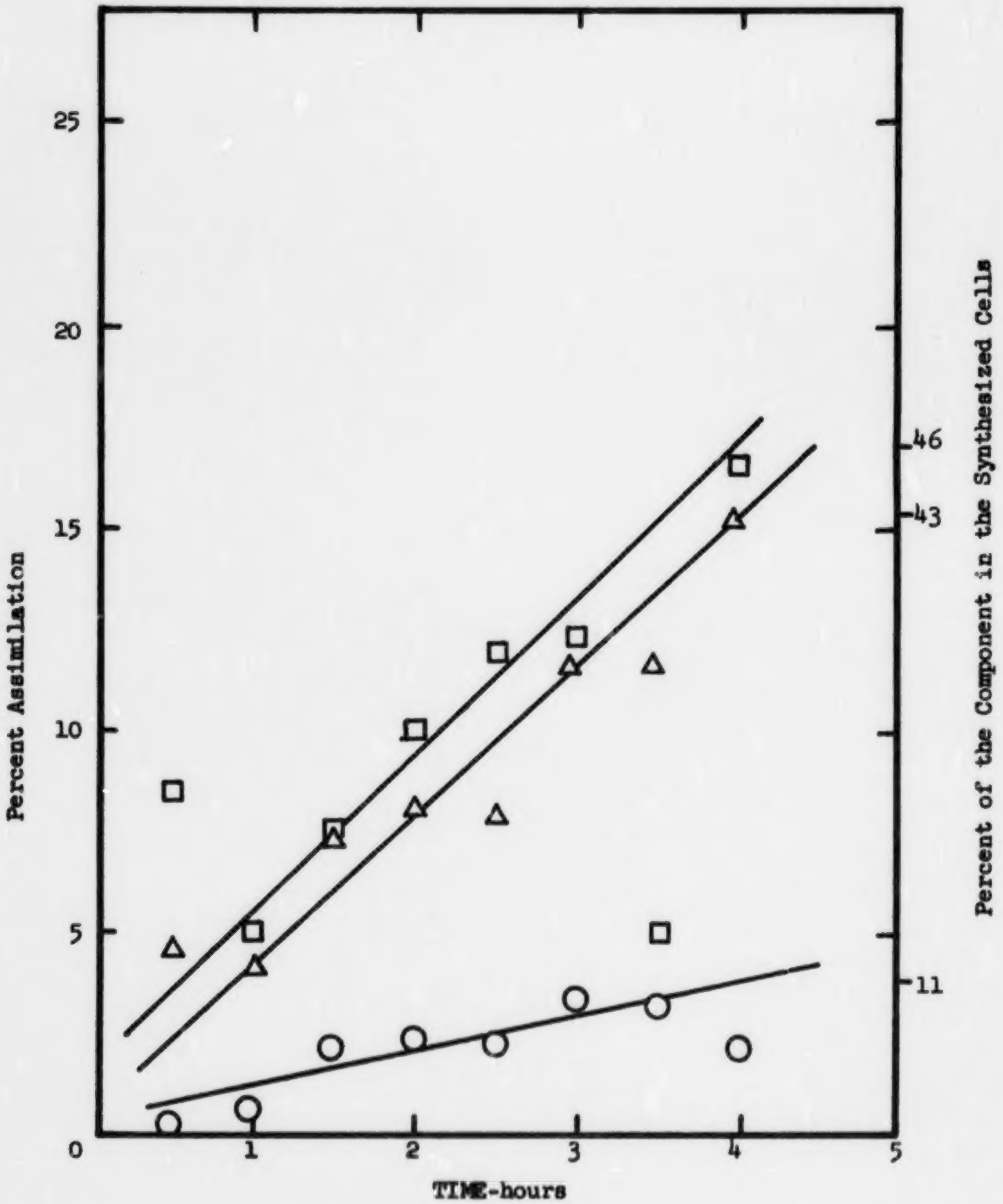


FIGURE 16 - SYNTHESIS OF ACETATE-2-C^{14} SLUDGE

BASIN #6



△ Proteins ○ Fats □ Carbohydrates

FIGURE 17 - SYNTHESIS OF ACETATE-2-C^{14} SLUDGE

BASIN #7

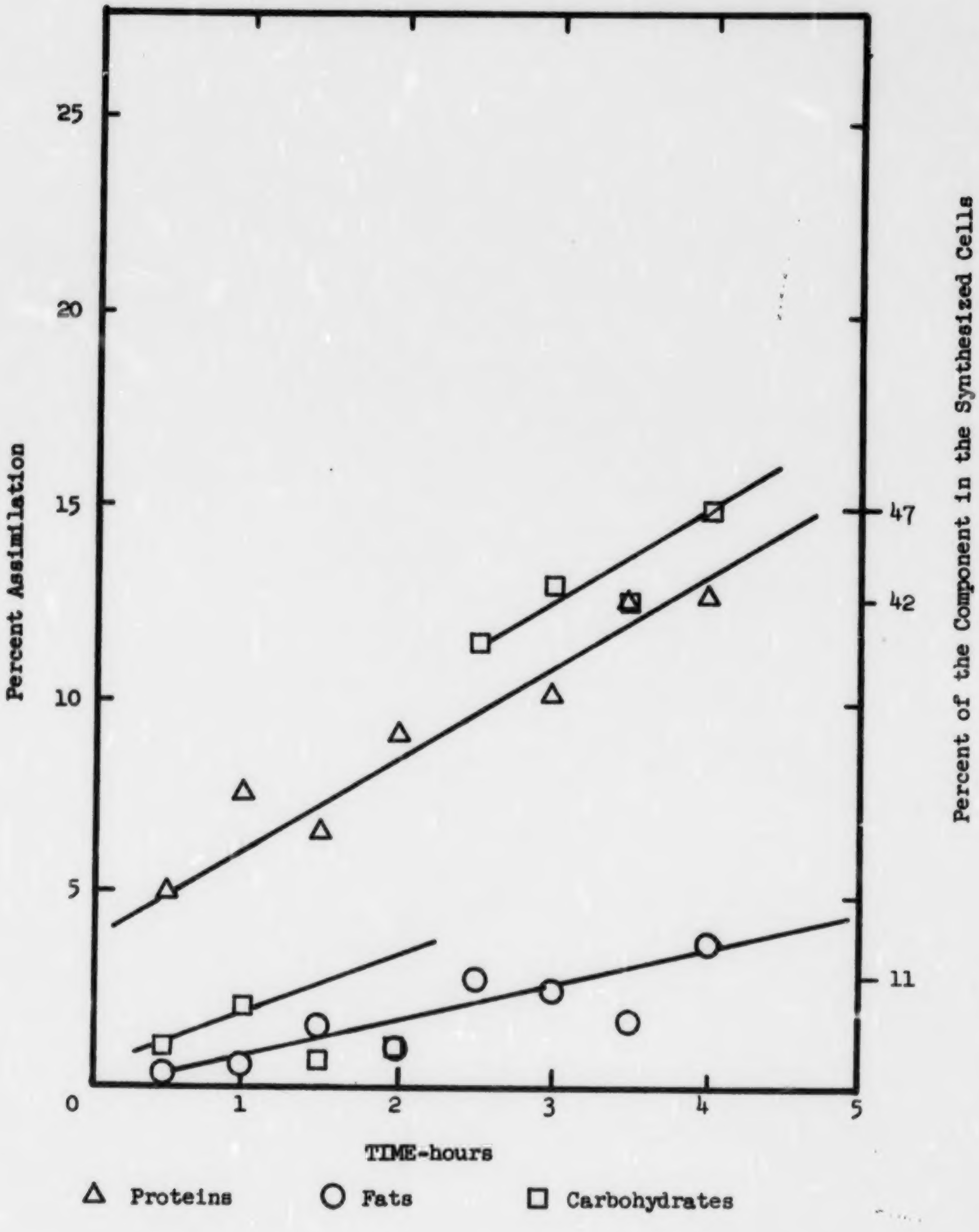


FIGURE 18 - SYNTHESIS OF ACETATE-2-C^{14} SLUDGE

BASIN #8

of the per cent composition of the synthesized sludge given above were made, as in the previous investigation, on the basis of the sodium acetate assimilated through the second hour. The calculation for the fill and draw systems were based on the per cent assimilation four hours after inoculation of the radioactive substrate. The four hour determinations were chosen for this purpose for it appeared that the fill and draw systems took at least this long to metaboliz the fed substrate. This was evident from the fact that the per cent assimilation did not level off over the period of test.

It was noted from the synthesis curves (Figures 15-18) that the carbohydrate fractions gave higher apparent synthesis values than the proteins in all systems. As was explained in detail in the Acetate-1-C^{14} section, the major reason for the apparent carbohydrate synthesis being larger than that in synthesized sludge was that the sludge contained a substantial proportion of biologically inactive external polysaccharides.

The accumulation of external polysaccharides in the continuous flow systems did not entirely explain the reason why the apparent carbohydrate synthesis was slightly higher in the flow through basins than in the batch basins for the total volatile solids and carbohydrates were still higher in the batch systems than in the continuous flow systems. An additional boost in the apparent carbohydrate synthesis resulted from the use of methyl carbon labeled acetate. As a result of the synthesis pathways for acetate, the synthesized glucose, and consequently the polysaccharides, contains about 33 per cent more carbon-14 than the fed substrate. This selective assimilation of methyl carbon into the carbohydrates increased the specific activity of the carbohydrate fraction of the continuous flow systems to a greater extent than that of the batch systems because of the fact that, even though the solids had increased over the Acetate-1-C^{14} units, the flow through systems had a much higher proportion of active mass than did the batch systems.

The above two discussed factors sufficiently influenced the radioisotope assimilation to yield the unexpected data. It should then be kept in mind that the above reported values for carbohydrates are in error.

The synthesis of protein in the acetate-2-C^{14} systems is shown in Figure 19. In general, the per cent of the substrate assimilated show fair agreement. Between all the units there is only about 3 per cent difference in protein

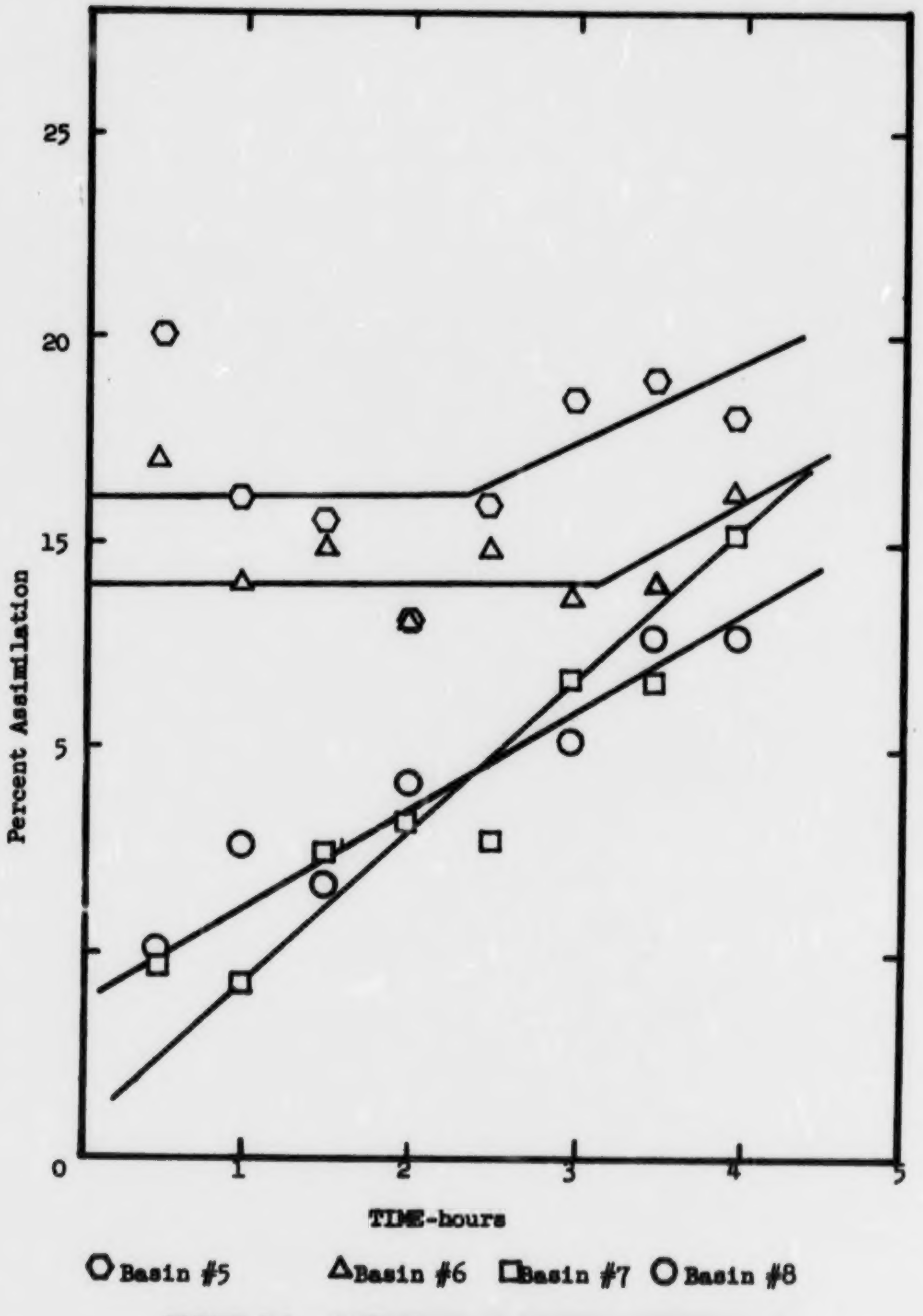


FIGURE 19 - COMPARISON OF PROTEIN SYNTHESIS IN ACETATE-2-C^{14} SYSTEMS

assimilation. The data were somewhat scattered, but even so, as shown by the curves, the duplicate samples of each type of unit showed similar assimilation patterns.

Autolysis: The activated sludge remaining in the basins after the synthesis phase of the investigation was used to obtain information on the degradation rates of the various cell components. Samples were taken and chemically analyzed for the sludge components of total volatile solids, protein, fat and reducing sugar. For comparative purposes, the components in each system as a per cent of the original volatile solids in the system have been plotted in Figures 20 through 23. Note that in Figure 20 the protein and fat data are exchanged.

The reliability of the analytical techniques are in evidence by the smooth transitional nature of all the curves. The general pattern of autolysis in both the continuous flow and the fill and draw systems was very similar. This similarity is emphasized on comparison of the degradation of volatile solids for each of the basins (Figure 24). The protein degradation accounts for the majority of the volatile solids change over the 27 days of the study. The sludge fat was reduced considerably in all basins; however, since they comprise such a small portion of the total solids their reduction has only minor influence on the total solids reduction. The reducing sugars content of all the sludges shows little or no reduction in measured quantity over the 27 day period. On the contrary, the reducing sugars in the fill and draw system show some inclination to even higher values of per cent of current volatile solids at the end of the period of investigation.

A better picture of the effects of autolysis on the sludge mass was obtained by presenting the data in graphs of the composition of current volatile solids, i.e., the sludge composition at any given time during the autolysis phase. The presentation of the autolysis data for each basin is given in Figures 25 through 28. The ordinate of the graphs represents 100 per cent of the sludge composition at any given time. The upper portion of the ordinate is the protein to which is added the fats. The fat percentages were added to the protein curve to avoid misleading variation in the fat curve as a result of poor protein determinations. The lower portion of each ordinate by difference represents the carbohydrate fraction of the sludge. The per cent of the volatile solids measured as reducing sugars has also been plotted to readily show the portion of the carbohydrate fraction which these analyses measured. These plots again show the striking resemblance between the degradation patterns of activated sludges grown in the continuous flow and fill and draw systems.

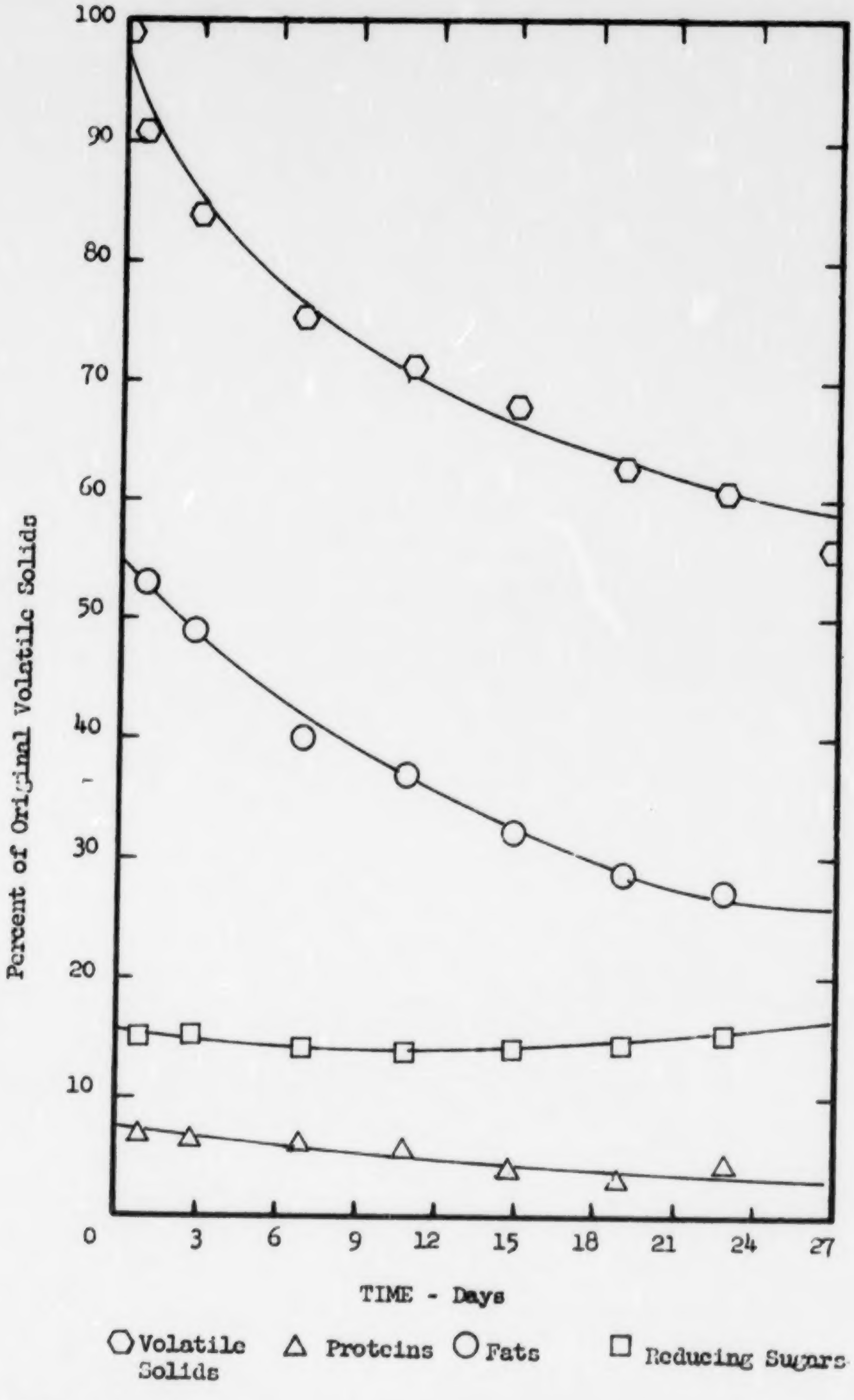


FIGURE 20- COMPONENTS AS PERCENT OF ORIGINAL VOLATILE SOLIDS

BASIN #5

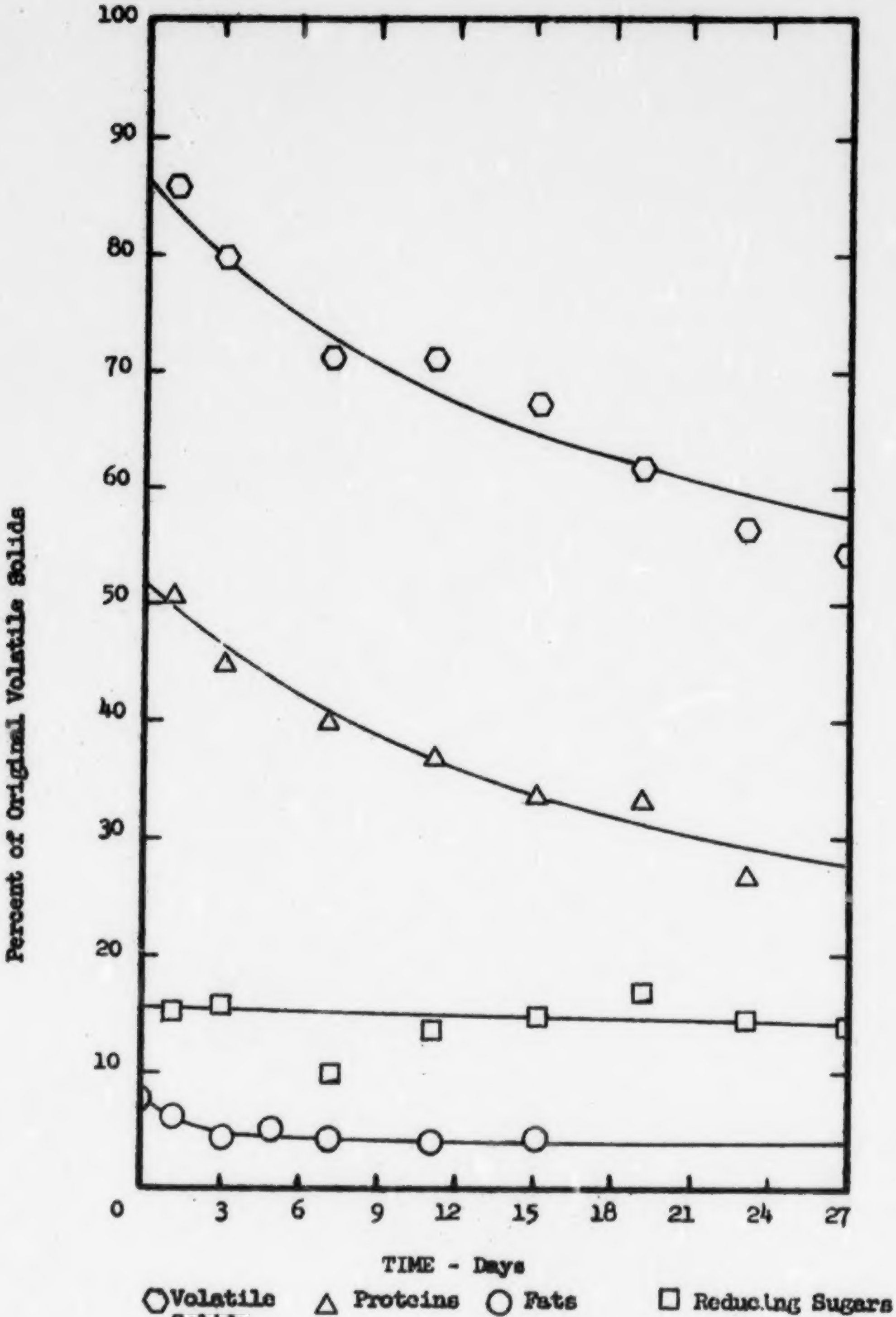


FIGURE 21 - COMPONENTS AS PERCENT OF ORIGINAL VOLATILE SOLIDS

BASIN #6

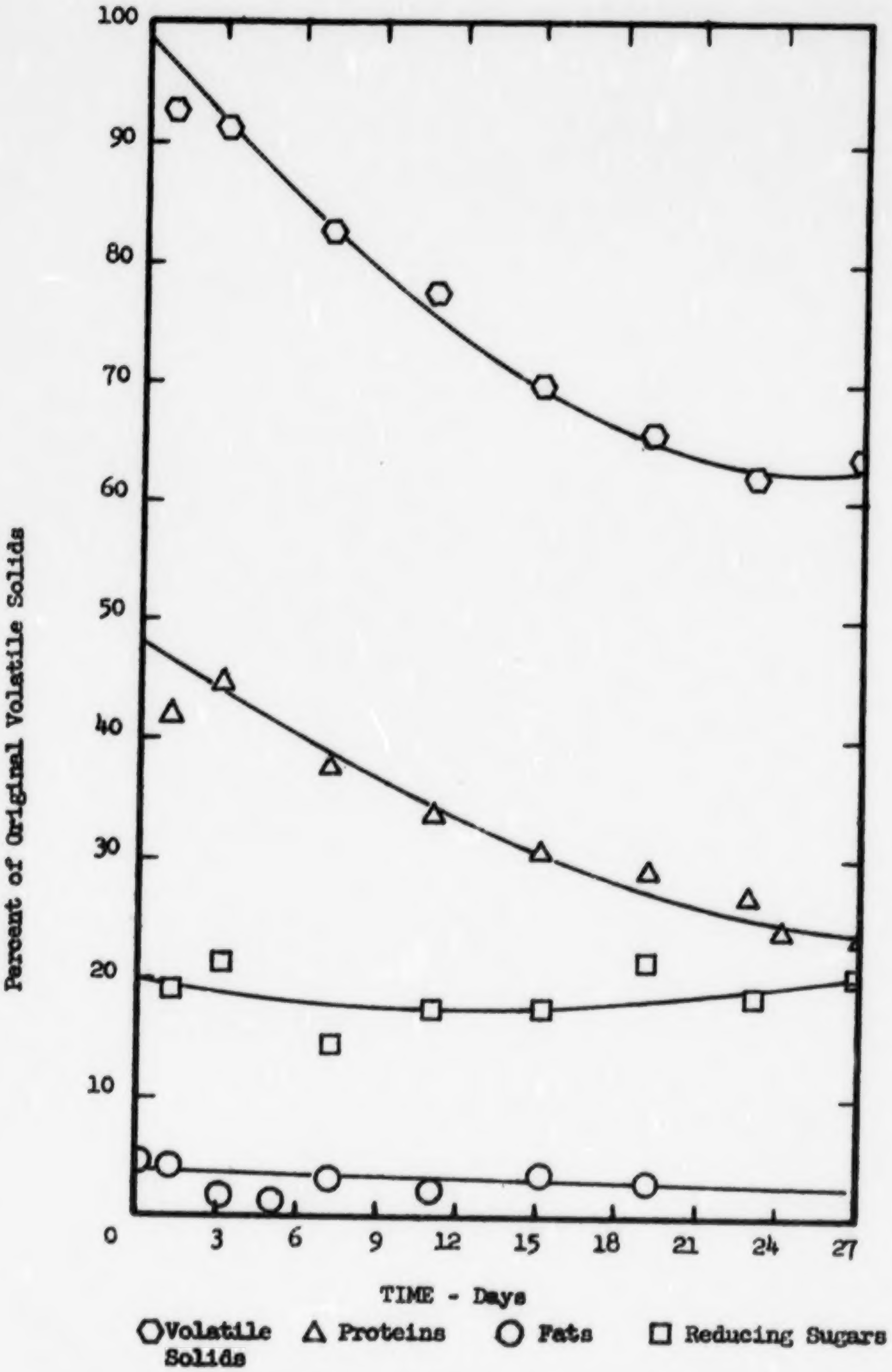


FIGURE 22 - COMPONENTS AS PERCENT OF ORIGINAL VOLATILE SOLIDS

BASIN #7

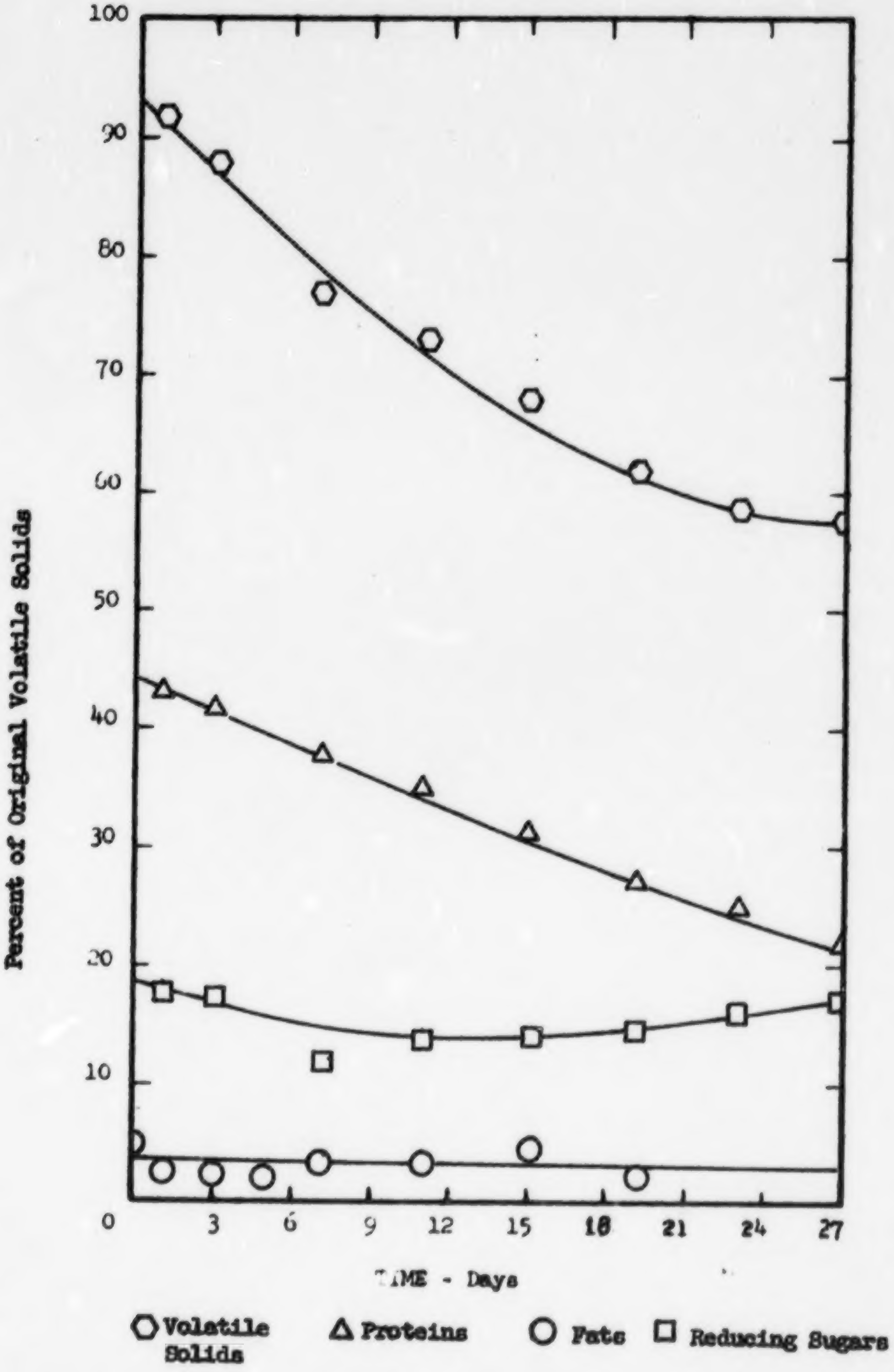


FIGURE 23 - COMPONENTS AS PERCENT OF ORIGINAL VOLATILE SOLIDS

BASIN #8

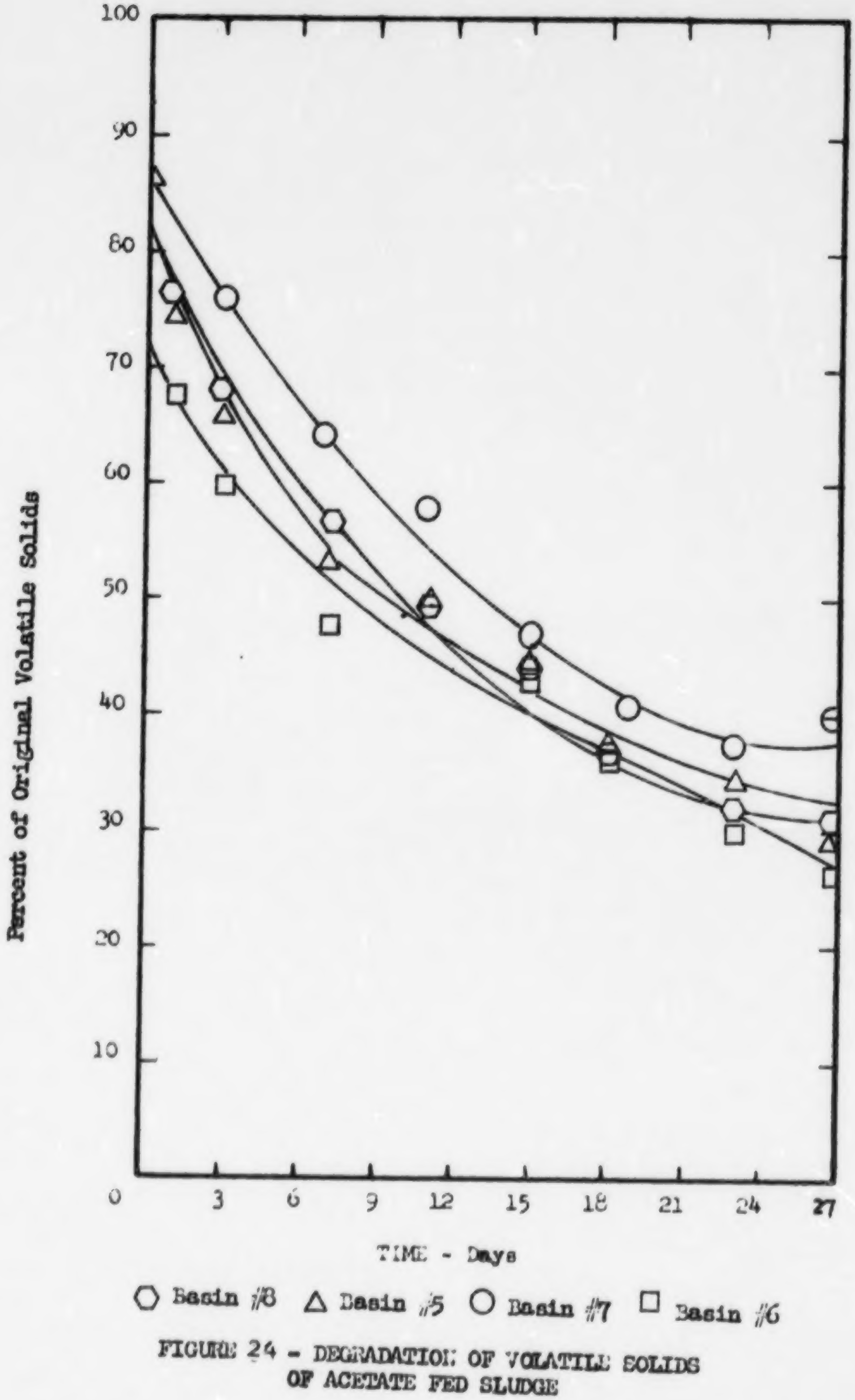


FIGURE 24 - DEGRADATION OF VOLATILE SOLIDS OF ACETATE FED SLUDGE

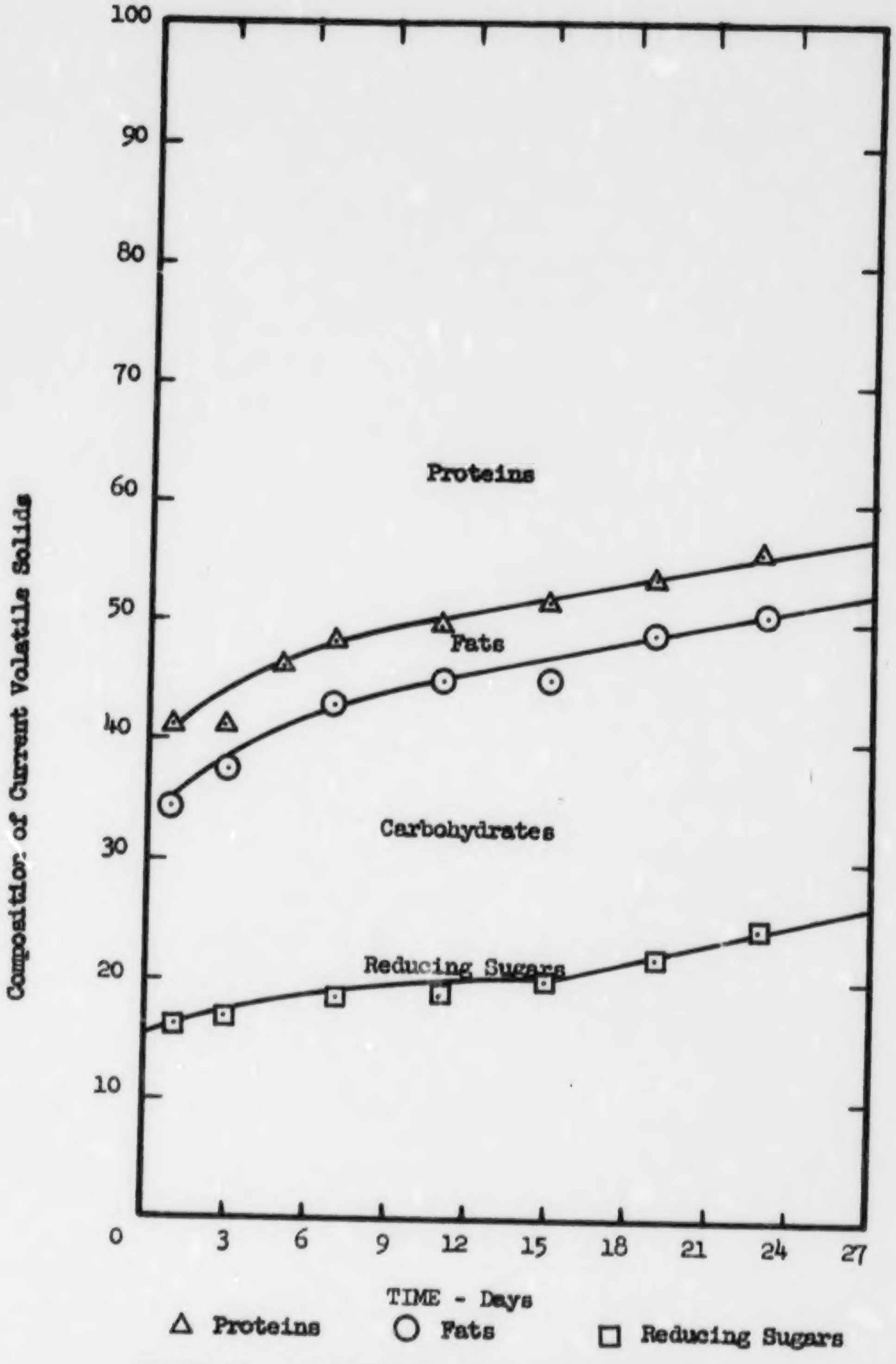


FIGURE 25 - COMPOSITION OF CURRENT VOLATILE SOLIDS

BASIN #5

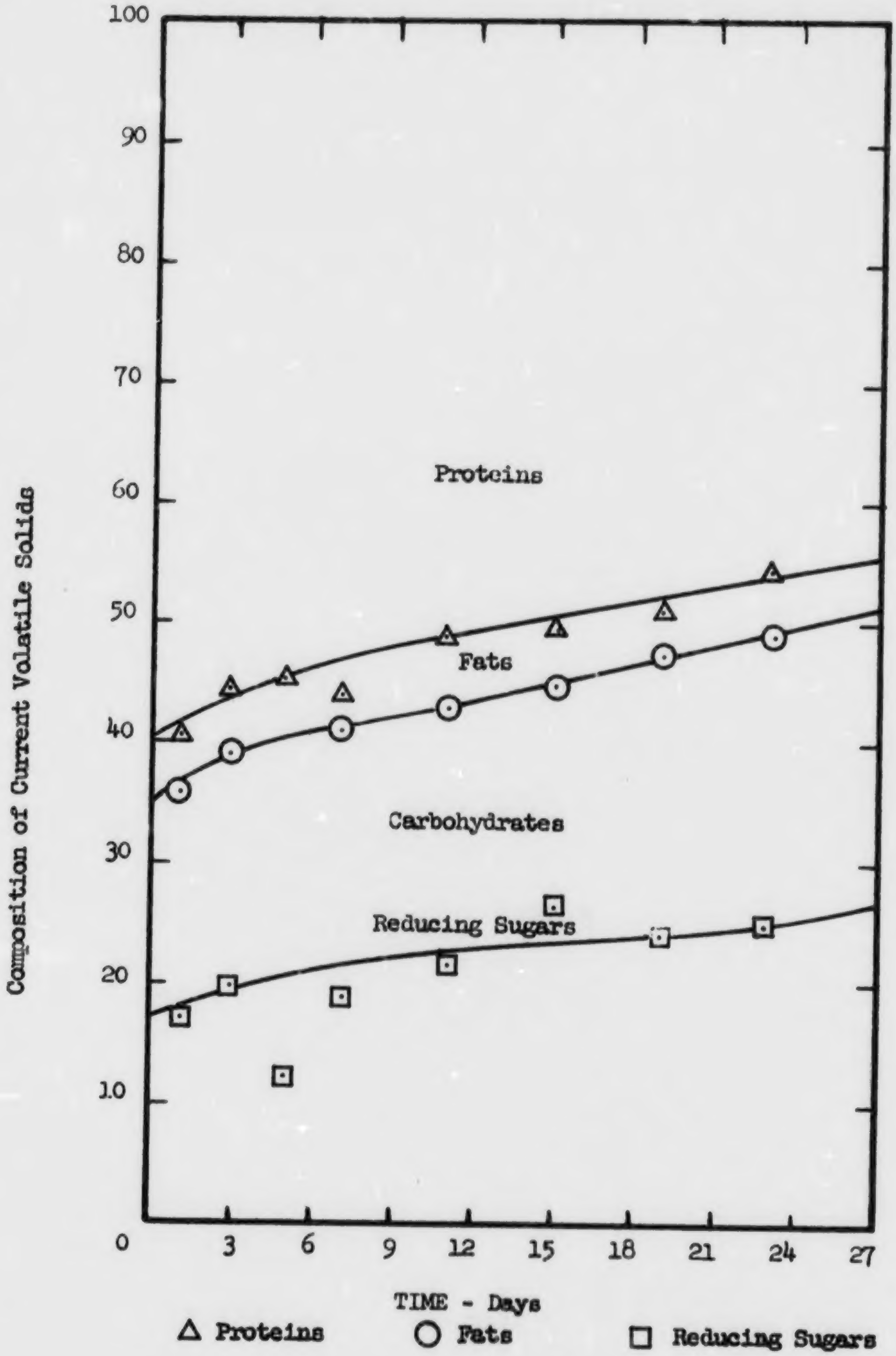


FIGURE 26 - COMPOSITION OF CURRENT VOLATILE SOLIDS

BASIN #6

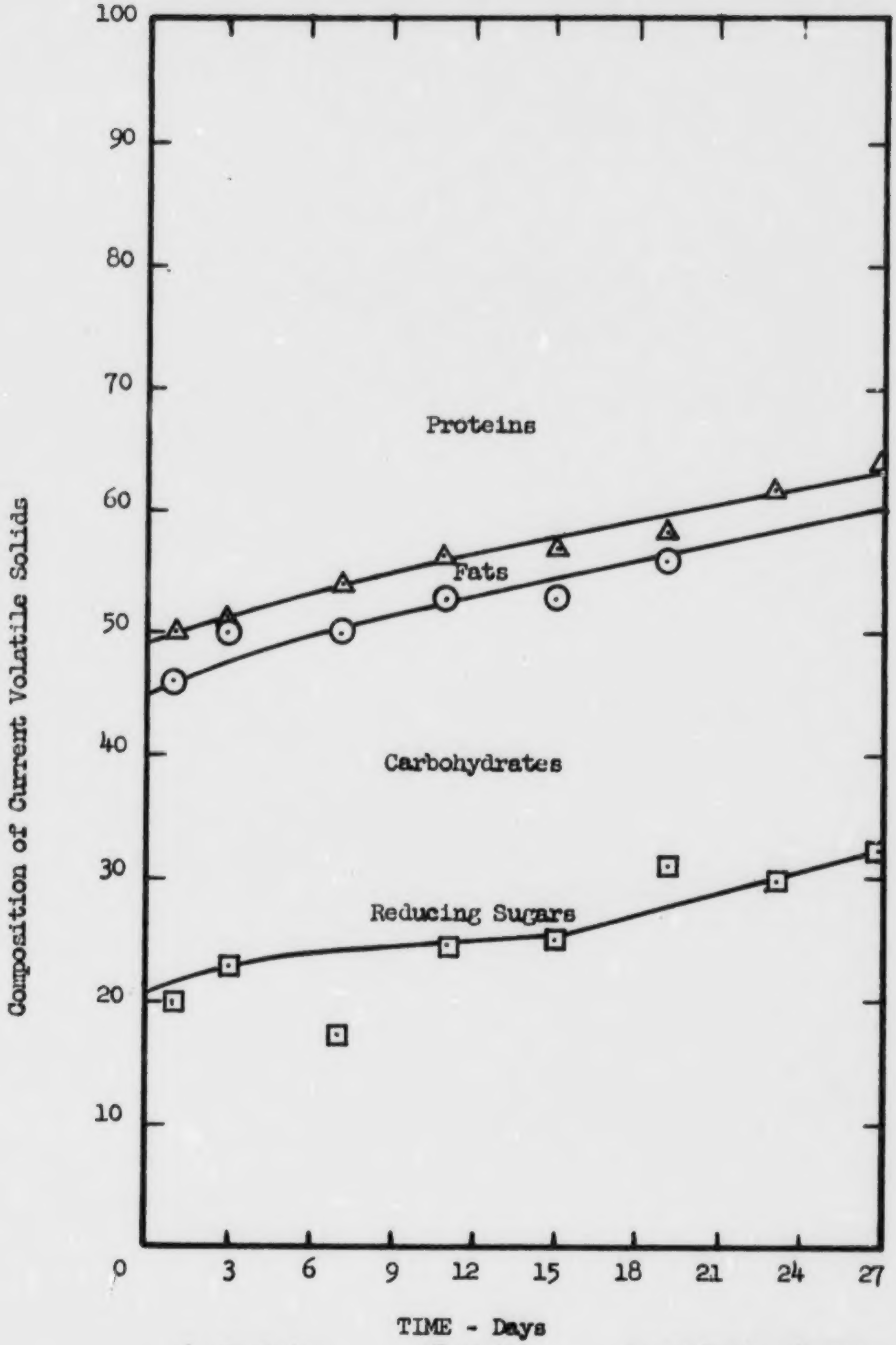


FIGURE 27 - COMPOSITION OF CURRENT VOLATILE SOLIDS

BASIN #7

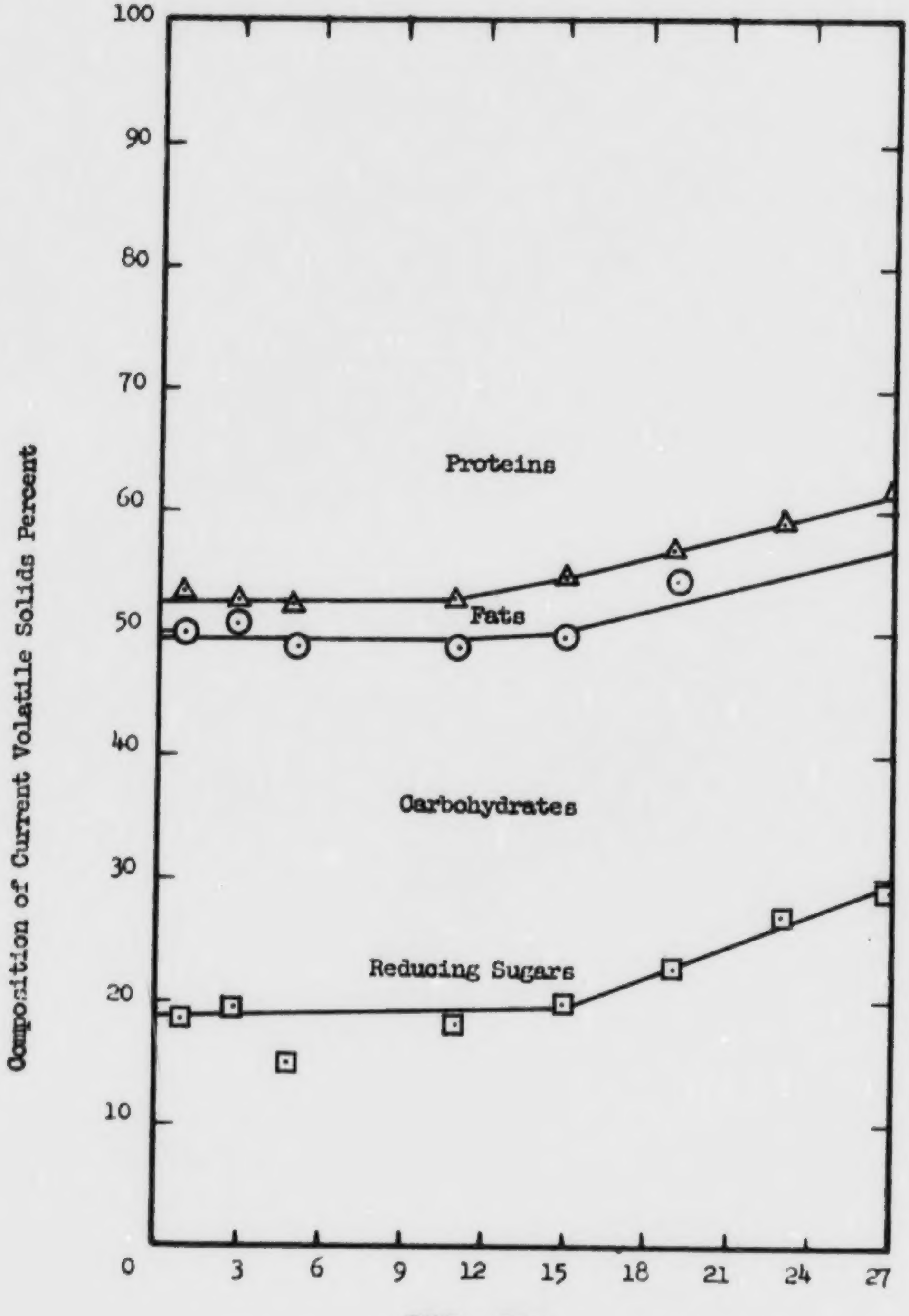


FIGURE 28 - COMPOSITION OF CURRENT VOLATILE SOLIDS

BASIN #8

These curves (Figures 25-28) show very clearly the fact that the carbohydrates compose a greater proportion of the sludge as endogenous respiration continues. Although it is not very discernible by viewing these curves, in the fill and draw systems, the reducing sugar analysis also measured an increasing per cent of the carbohydrates during the latter part of the autolysis period. To show the various relationships of the carbohydrate fractions, the data from Figures 20 through 28 have been summarized in Table XI. Carbohydrate and reducing sugar values for the original sludge and that on the third and also the last day of autolysis have been tabulated. The data from the third day was included to avoid any erroneous conclusions resulting from immediate degradation if it occurred.

In the continuous flow system, after an initial reduction in one system of about 3 per cent, the total weight of carbohydrates did not change for the duration of the study. The initial reduction of carbohydrates mentioned above was the result of degradation of the reducing sugar fraction which fell from 47 to 44 per cent of the original carbohydrates. After the third day, the reducing sugars measured a constant 49 per cent of the carbohydrates.

In the fill and draw systems, the sludges increased in carbohydrate content over the period of investigation. At the same time, however, the total amount of carbohydrate in the systems decreased considerably. In Basin #7 the carbohydrates fell from 43 to 38 per cent of the original volatile solids. In Basin #8 tje decrease was from 47 to 32 per cent.

Summary: The systems were operating well into the equilibrium phase prior to initiation of the start of the synthesis studies. The volatile solids in the continuous flow systems was, in fact, from 45 to 80 per cent higher than in the acetate-1-C^{14} investigation. The volatile solids in the batch systems averaged about 10 per cent higher. These higher sludge solids were represented for the most part in an increase in the carbohydrate fraction. This increase in carbohydrates was evidenced by the lower nitrogen concentration of the sludge mass.

The high carbohydrate was also reflected in the synthesis data. The per cent synthesis of both the protein and fat fractions for the respective feeding schedules were substantially the same as in the acetate-1-C^{14} systems. The apparent carbohydrate synthesis was considerably higher than would be expected for average sludge synthesis.

All systems showed striking similarity in their degradation patterns. During autolysis the protein in all the acetate-2-C^{14} sludges decreased more rapidly as a per cent of the total sludge than the other cell components. The protein was removed as an exponential fraction of the amount present which resulted in a trend to leveling off at the end of the 27th day of the autolysis period after about 40 to 50 per cent of the protein was removed. The fats were removed only over the initial endogenous respiration period after which they declined only slightly over the remainder of the experiment. The carbohydrates showed a small initial decrease with a subsequent leveling off which indicated that these fractions were not easily degraded. This was shown also by the continuous increase in the per cent of the current sludge represented by the carbohydrate fractions.

D. Glucose-U-C^{14} Systems

Having completed the study of activated sludge grown on acetate, the next substrate used in the investigation was glucose. As mentioned earlier, glucose was chosen to represent carbohydrate containing wastes.

Carbon Balance: The carbon balances for all the systems were started on the seventh week of growth. The first balance was completed on the ninth week. The carbon balance determinations were continued through the twelfth week. The summary of the carbon balances for the continuous flow systems #9 and #10 is presented in Table XII. The summary of the carbon balances for the fill and draw systems #11 and #12 is given in Table XIII.

The carbon balances for all the glucose-u-C^{14} systems yielded good results with only one closing discrepancy exceeding 8 per cent. The highest closing discrepancy was that of 10 per cent for the tenth week balance on the continuous flow system #9.

The solids in the fill and draw systems accumulated to about 7,000 mg/l. Even so, it was not necessary to practice sludge wasting on the 50 per cent return operational procedure. The sludges were dense and settled within the hour to less than half the basin capacity. The solids in the continuous flow systems were about 5,000 mg/l for Basin #9 and 4,500 mg/l for Basin #10. It will be noted that there was not as great a difference between the continuous flow system solids and those in the fill and draw systems as had been experienced in the previous acetate experiments. This situation arose as a

TABLE XII

SUMMARY OF CARBON BALANCE FOR CONTINUOUS FLOW BASINS - UNIFORM LABELED GLUCOSE

1	2	3	4	5	6	7	8	9
Week in Basin	Carbon Removed mg	CO_2-C Recovered mg	Cell Growth-C mg	Effluent Cells-C mg	Cells-C Removed during Analysis mg	Balance mg-C	Closing[1] Per Cent	Per Cent Oxidized
System 9 - Continuous Flow								
7								
9	5250	4185	950	52	113	-70	1.3	80
10	3180	2765	560	61	100	-310	10	87
12	5140	5135	-580	45	113	350	6.8	100
System 10 - Continuous Flow								
7								
9	5250	4035	340	468	122	230	4.4	77
10	3175	2570	-10	596	90	-65	2.2	81
12	5190	4565	-130	460	410	-100	2.0	90

1. Calculated based on total substrate added.

TABLE XIII

SUMMARY OF CARBON BALANCE FOR CONTINUOUS FLOW BASINS - UNIFORM LABELED GLUCOSE

1	2	3	4	5	6	7	8	9
Week in Basin	Carbon Removed mg	CO_2-C Recovered mg	Cell Growth-C mg	Effluent Cells-C mg	Cells-C Removed during Analysis mg	Balance mg-C	Closing[1] Per Cent	Per Cent Oxidized
System 9 - Continuous Flow								
7								
9	5250	4185	950	52	113	-70	1.3	80
10	3180	2765	560	61	100	-310	10	87
12	5140	5135	-580	145	113	350	6.8	100
System 10 - Continuous Flow								
7								
9	5250	4035	340	468	122	230	4.4	77
10	3175	2570	-10	596	90	-65	2.2	81
12	5190	4565	-130	460	410	-100	2.0	90

---

[1] Calculated based on total substrate added.

result of the low and sometimes even negative cell growth which occurred in the fill and draw basins during the last few weeks of the carbon balance.

According to the per cent stabilization shown in the last column of the Summary of Carbon Balance Tables XII and XII, all the glucose fed sludges were operating at equilibrium by the end of this phase of the experiment. During the final balance from the tenth to twelfth week of operation, the continuous flow basin #9 activated sludge showed complete stabilization of the substrate as did the fill and draw basin #12 activated sludge over the same period. These periods of complete stabilization together with the high stabilization obtained in the balances for basin #11 strongly proves that the decline in sludge growth, mentioned above, was not the result of loss of sludge in the effluent. Rather the loss was the result of autolysis of the sludges.

Synthesis: The systems were made radioactive by the introduction of glucose-u-C^{14} labeled substrate diluted with carrier glucose. Graphical presentations of the synthesis in the various cell components are given in Figures 29 to 32.

As can be seen from Figures 29-32, the per cent of the substrates assimilated shows a somewhat different curve pattern than was obtained for the acetate fed sludges. This was especially so of the apparent carbohydrate assimilation which ran between 75 and 80 per cent of the fed carbon in the continuous flow systems and between 60 and 70 per cent in the fill and draw systems over the initial hours of metabolism. It was expected that the carbohydrate data would give high apparent synthesis in the fill and draw systems for the uniformly labeled glucose substrate which of course could not be distinguished in the carbohydrate fraction from the sludge carbohydrate derivatives. This would also be true of the continuous flow systems if they did not use all of the applied substrate in the period between inoculation or, more likely, if the sludge had rapidly accumulated stored carbohydrate in the form of glycogen. It is obvious that it would take only a small amount of the very high specific activity glucose or glycogen in the carbohydrate fraction to markedly increase the apparent carbohydrate assimilation. The high carbohydrate content of the sludges must have had some effect on the increased assimilation as was shown in the acetate systems. This factor was, however, not of unmetabolized substrate in the mixed liquor or storage.

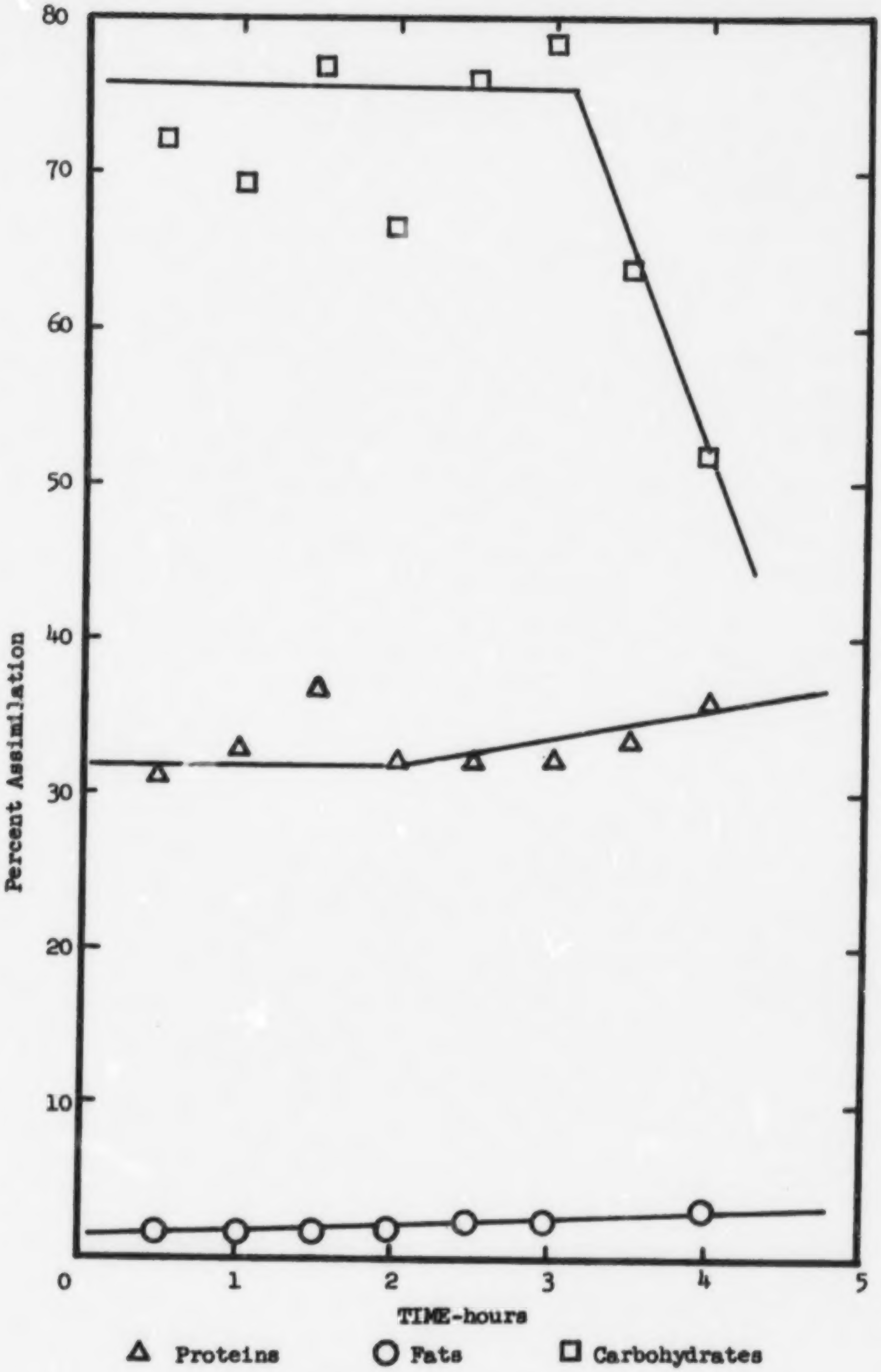


FIGURE 29- SYNTHESIS OF GLUCOSE SLUDGE

BASIN #9

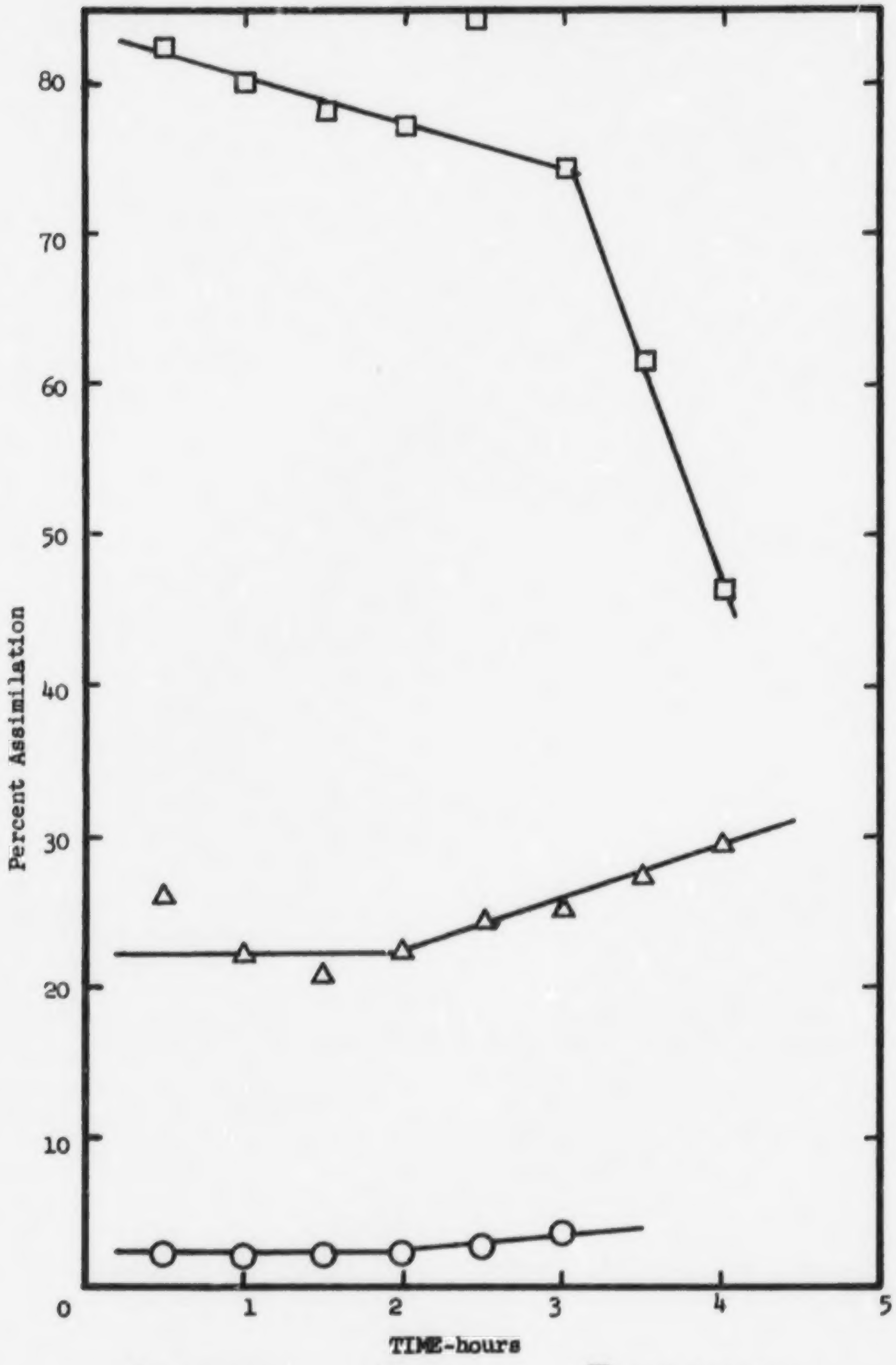


FIGURE 30 - SYNTHESIS OF GLUCOSE SLUDGE

BASIN #10

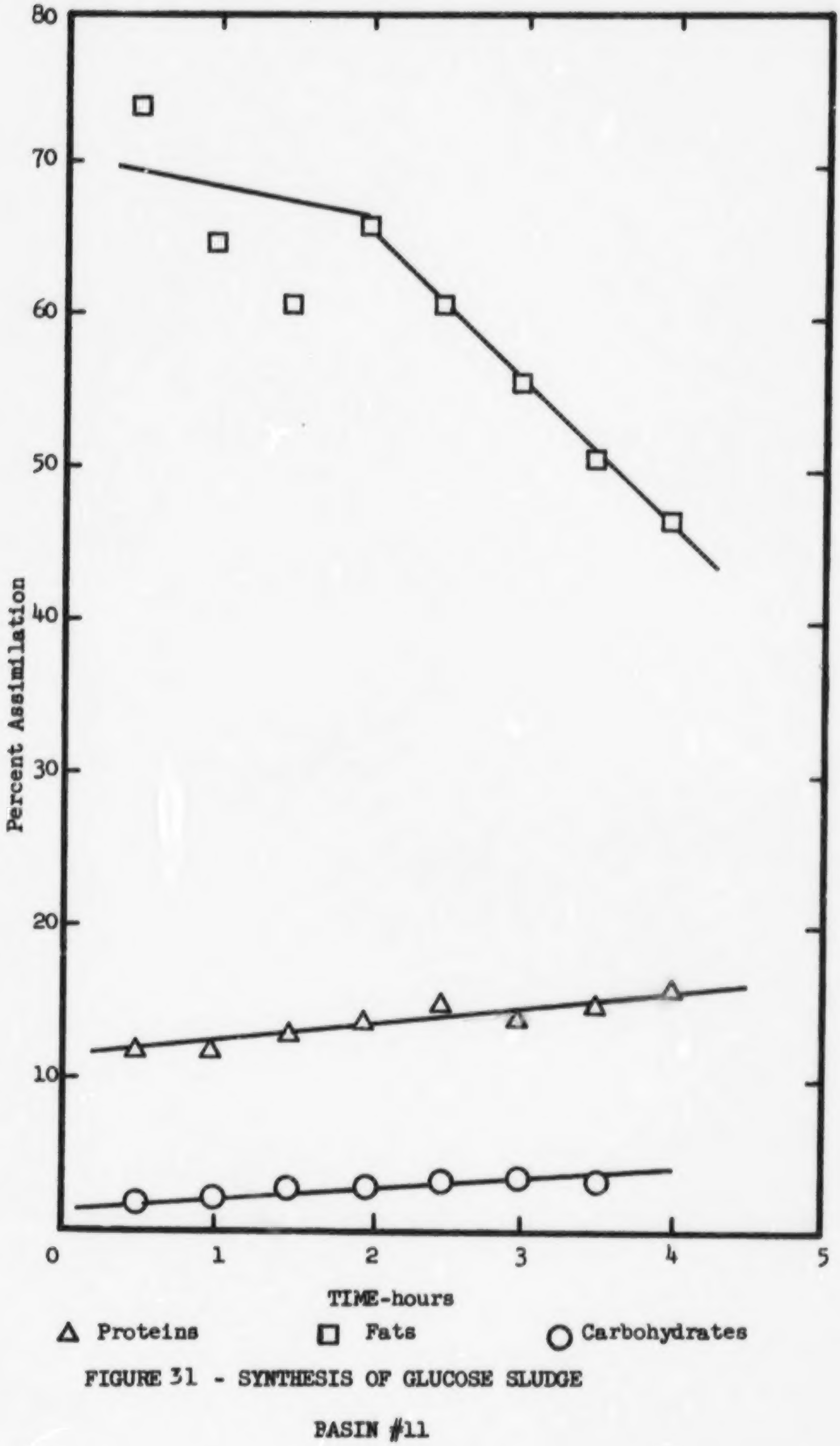
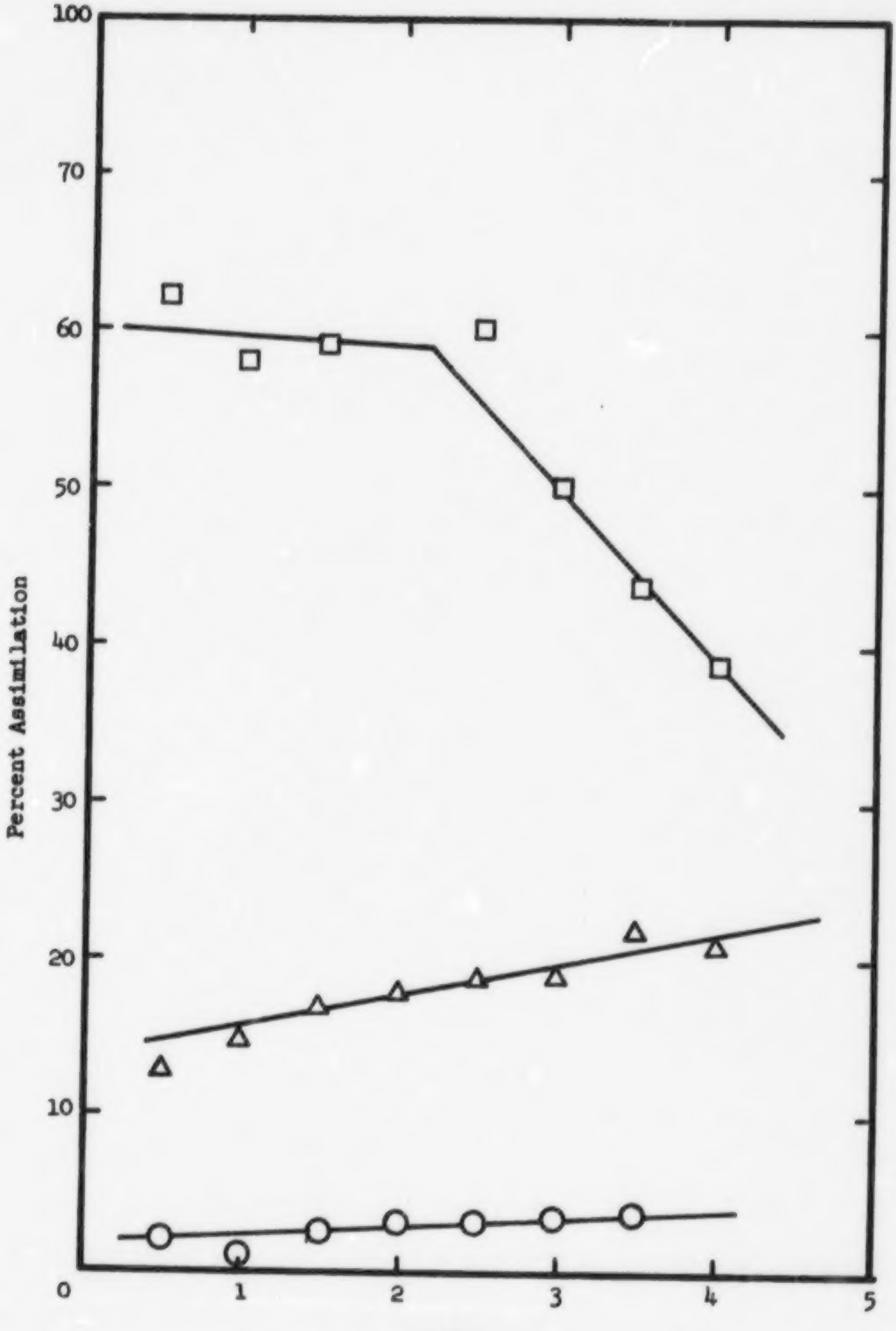


FIGURE 31 - SYNTHESIS OF GLUCOSE SLUDGE

BASIN #11



△ Proteins ○ Fats □ Carbohydrates

FIGURE 32 - SYNTHESIS OF GLUCOSE SLUDGE

BASIN #12

The fact that the apparent carbohydrate synthesis dropped rapidly between the second and third hours strongly supports the contention that a portion of the substrate was being stored as glycogen. As was mentioned above, it would take only a small quantity of substrate to markedly increase the specific activity of the carbohydrate fraction. Once the substrate was utilized, the stored material was then metabolized. Had the radioactive carbon been uniformly distributed throughout the active cell, the specific activity would not have fallen measureably during the four hours of the test as was the case in the acetate systems. As it was, the specific activity fell sharply indicating the rapid metabolism of a highly active substance.

As shown by the protein and fat curves, there were no rapid increases in these components over the period of decline in the apparent carbohydrates. This would not have been the case had the decline been the result of metabolism of a sizeable portion of the substrate.

The protein in the continuous flow systems again may have showed carbon dioxide incorporation after the feeding period ended. The total amount of possible incorportation was less than in the previous studies. The reason for the lower value was probably due to a difference in the bacterial species present in the basins, but no laboratory evaluation was made to confirm this.

Since the calculated carbohydrate assimilation was not the true value in any systems and the fats and proteins showed a continued rise through the fourth hour in the fill and draw no attempt was made to show the percentage composition of the newly synthesized cells. It was noteworthy, however, that the ratio of protein to fats in both continuous flow systems was about the same as that of the average bacteria.

The per cent of the glucose-u-C^{14} assimilated into the protein of the various glucose sludges is presented in Figure 33. This comparison shows that the synthesis in the fill and draw systems followed similar patterns. Also the continuous flow systems gave comparable results. The steady increase in protein synthesized, from the given amount of substrate, in the batch systems was a consequence of the slow utilization of the glucose in solution. The cause of the initial protein synthesis was not determined in this investigation.

Autolysis: The glucose sludge which remained in the basins after the synthesis phase of the investigation was used to obtain information on the degradation rates of the various cell components. The components in each system as a per

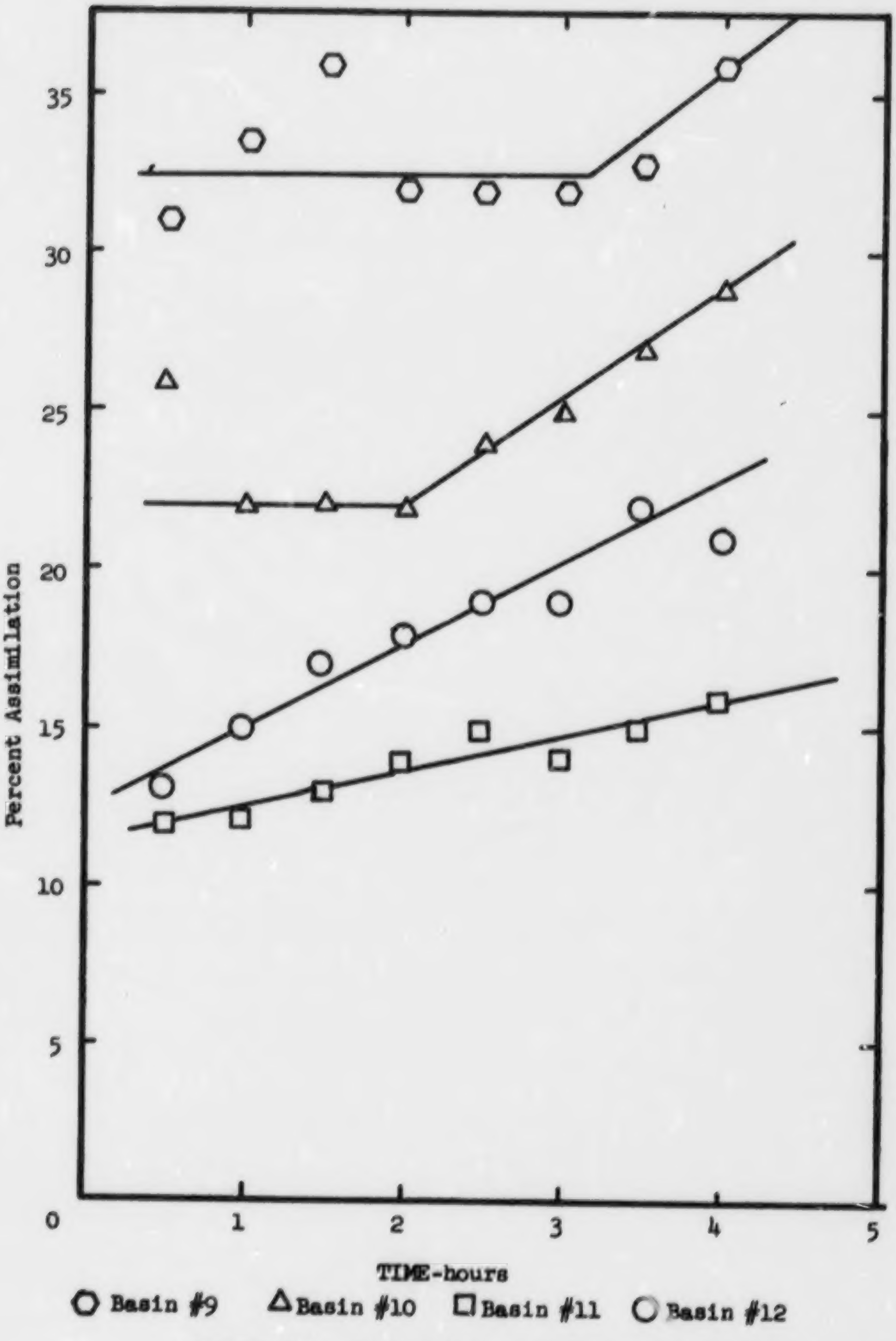


FIGURE 33 - COMPARISON OF PROTEIN SYNTHESIS IN GLUCOSE SYSTEMS

cent of the original volatile solids in the systems have been plotted in Figures 34 through 37.

As in the case of the acetate fed systems, the majority of the solids change was a result of protein autolysis over the 27 days of the study. With the glucose sludge, however, the initial solids reductions were also effected by the reduction in the carbohydrates and fats. The wider distribution of the inert material among the cell components in the glucose sludges was shown by the fact that the sludge protein accounted for less of the suspended volatile solids reduction in this experiment than in the previous studies with acetate. In the four glucose fed units of the sludge protein degradation amounted to from 54 to 62 per cent of the total volatile solids reduction with an average of 57 per cent. The total autolysis amounted to 63 to 78 per cent of the original volatile solids reduction with an average of 70 per cent. Another indication of the more uniform degradability of the glucose sludges was the reduction of 5 to 10 per cent of the suspended volatile solids over the first ten days of non-fed operation.

There is a striking similarity between these curves for all the systems, both flow through and batch type. This similarity is emphasized by comparison of the degradation of the volatile solids for each of the basins presented in Figure 38.

As inferred above, the residual activated sludge materials in the glucose grown systems were more widely distributed between the components than in the acetate sludges. This is clearly shown by the graphs of the composition of the current volatile solids, i.e., the sludge composition at any given time during the autolysis phase. These graphs for each system are given in Figures 39 to 42. The ordinate on a graph represents 100 per cent of the sludge composition at any given time. The upper portion of the graph is the proteins. The fats are added to the protein curve rather than the individual points to avoid misleading variation in the fat curve due to poor protein determinations. The lower portion of each ordinate by difference represents the carbohydrate fraction of the sludge. The per cent of the volatile solids measured as reducing sugars has also been plotted to readily show the portion of the carbohydrate fraction which these analyses measured. After the first three days, the composition of the sludge varied little. The per cent protein, for example, for all the activated sludges decreased from 2 to 6 per cent over the 27 days of observations. The carbohydrate, in most systems,

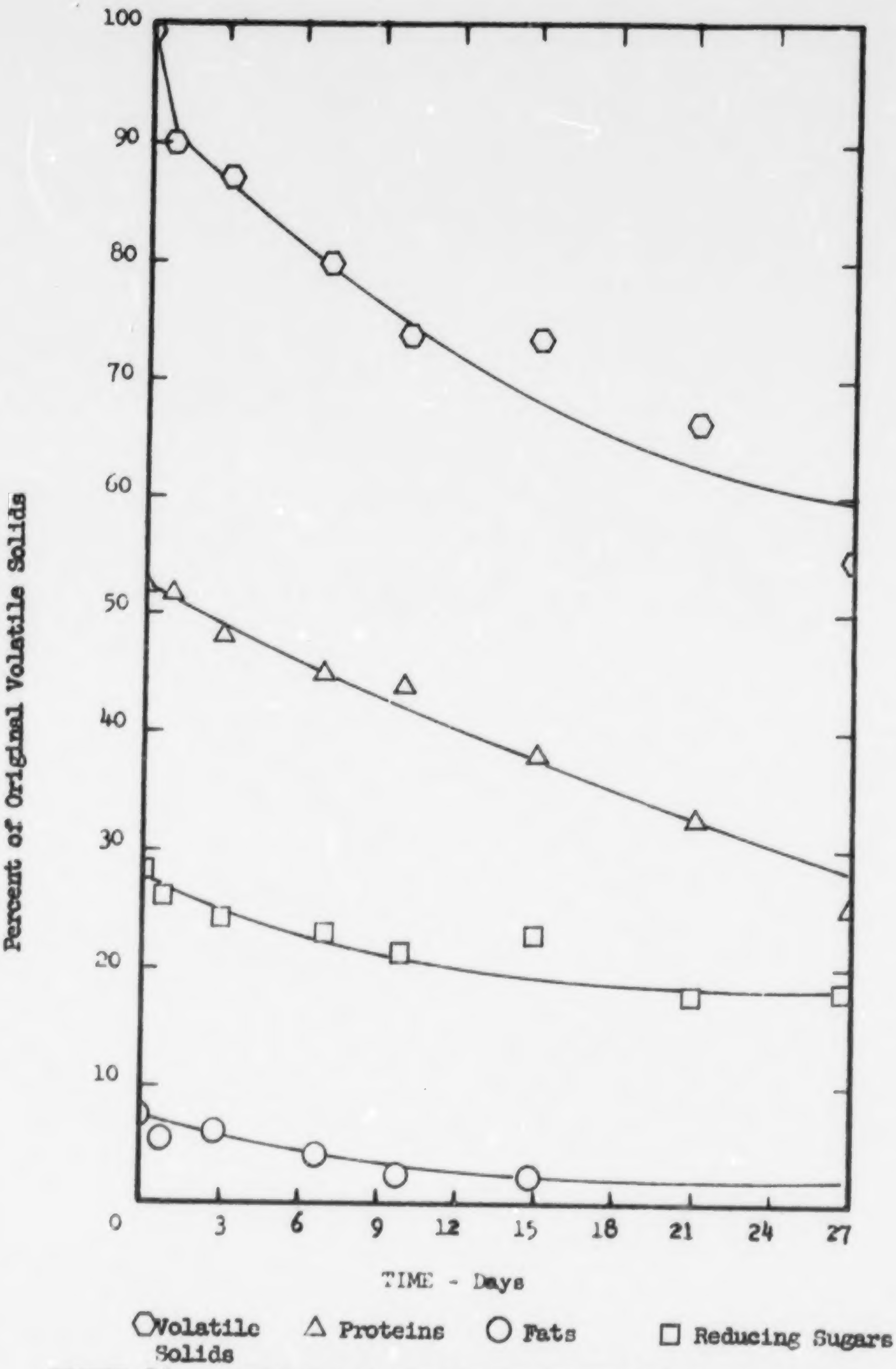


FIGURE 34 - COMPONENTS AS PERCENT OF ORIGINAL VOLATILE SOLIDS

BASIN #9

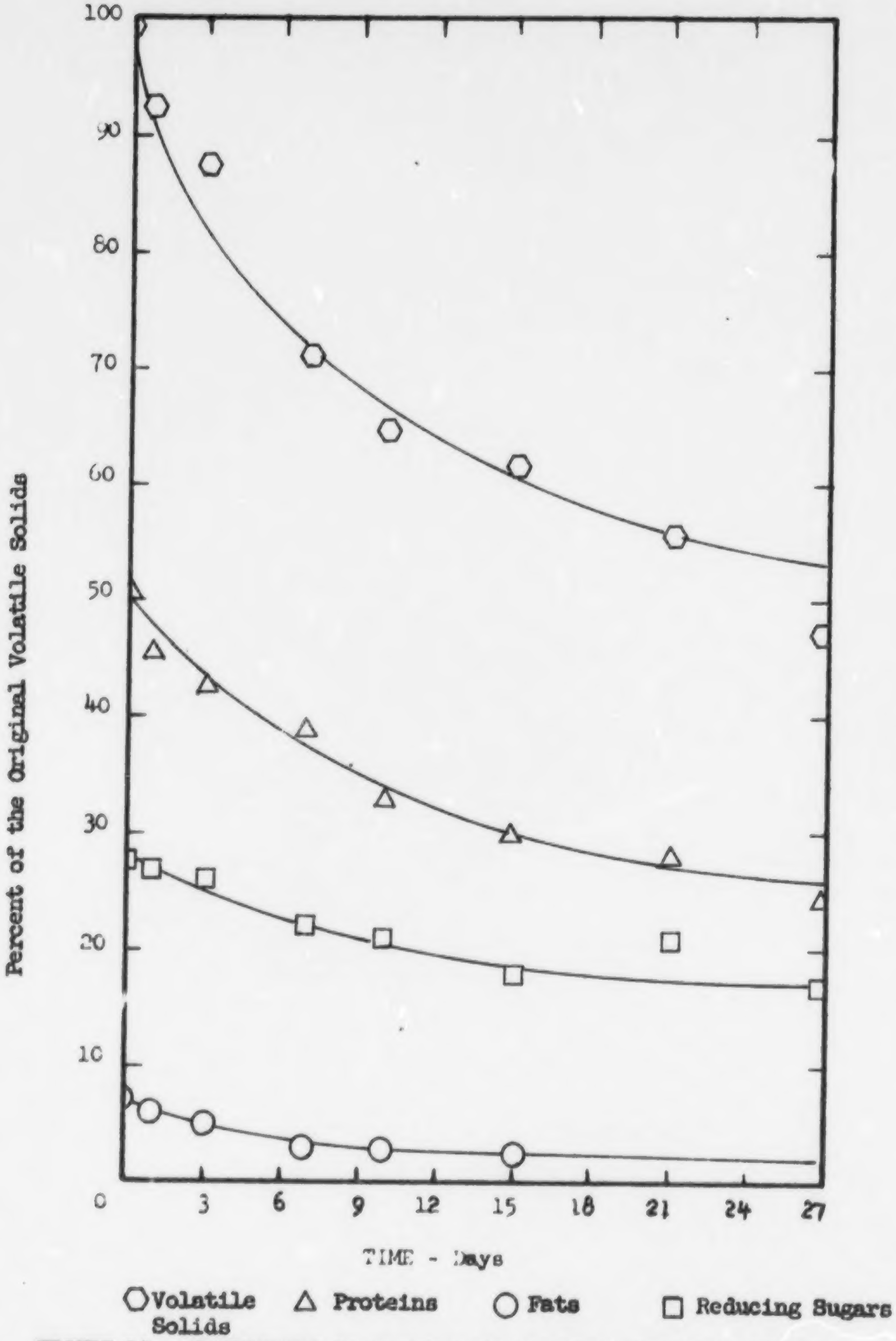


FIGURE 35 - COMPONENTS AS PERCENT OF ORIGINAL VOLATILE SOLIDS

BASIN #10

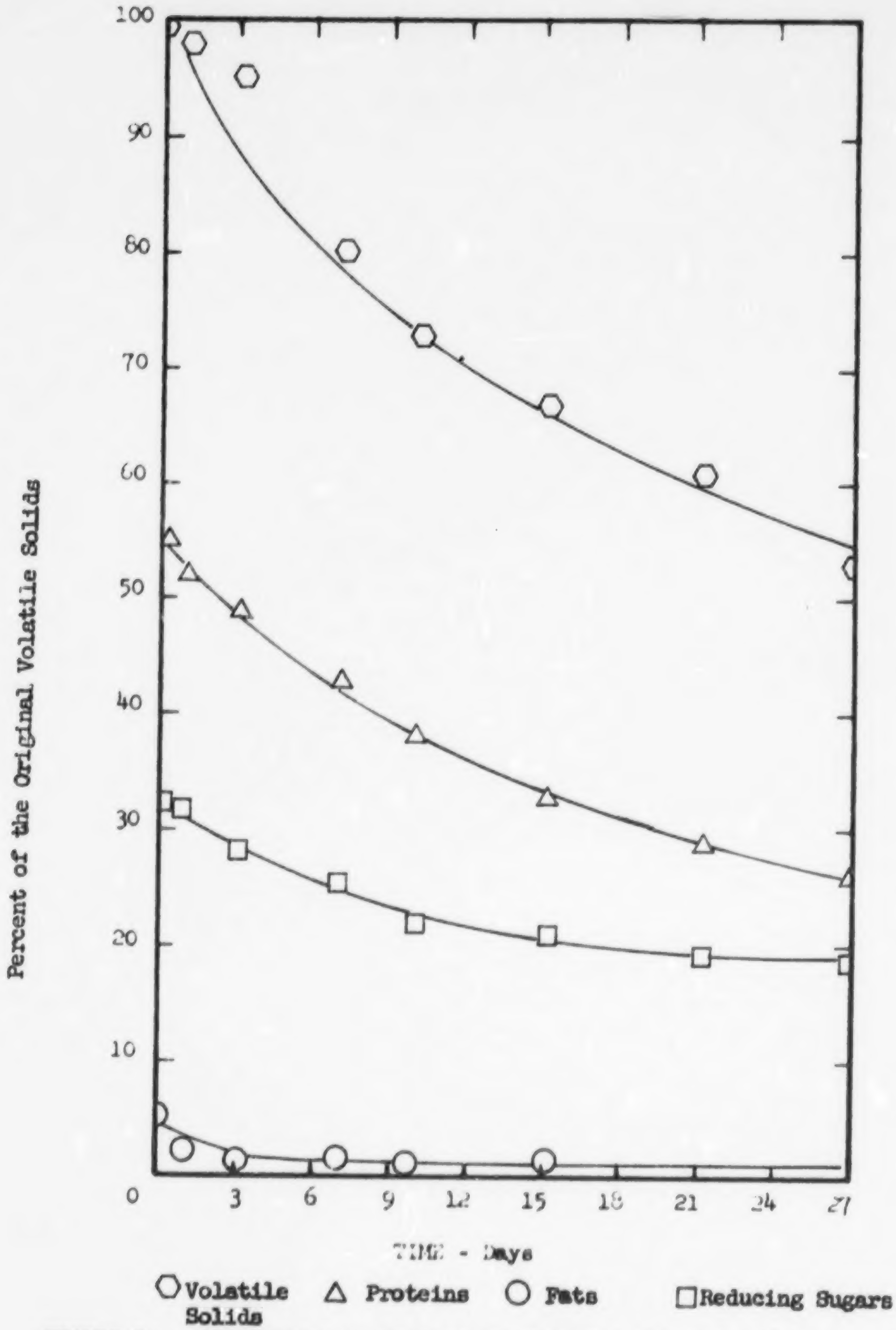


FIGURE 35 - COMPONENTS AS PERCENT OF ORIGINAL VOLATOLE SOLIDS

BASIN #11

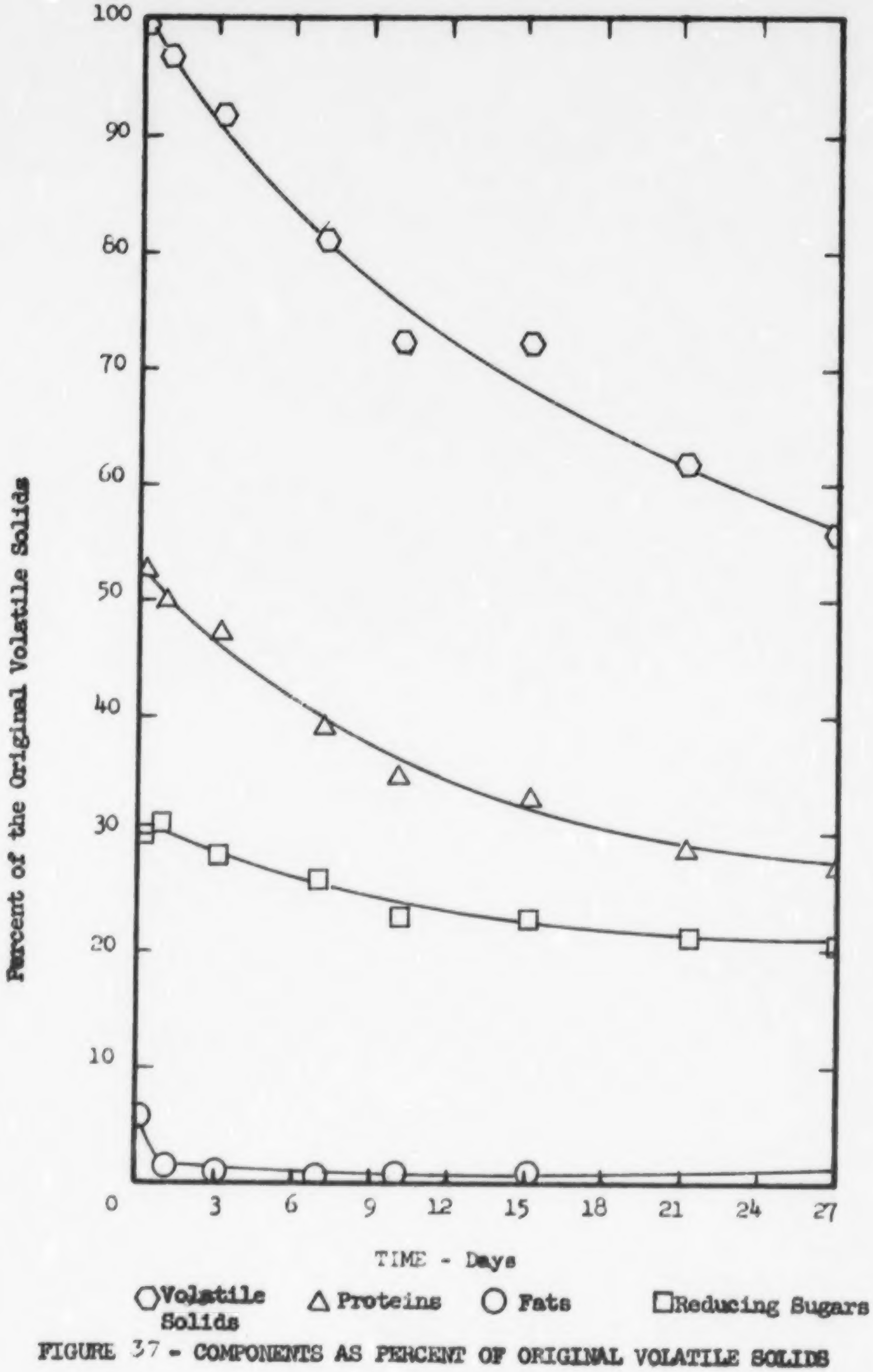


FIGURE 37 - COMPONENTS AS PERCENT OF ORIGINAL VOLATILE SOLIDS

BASIN #12

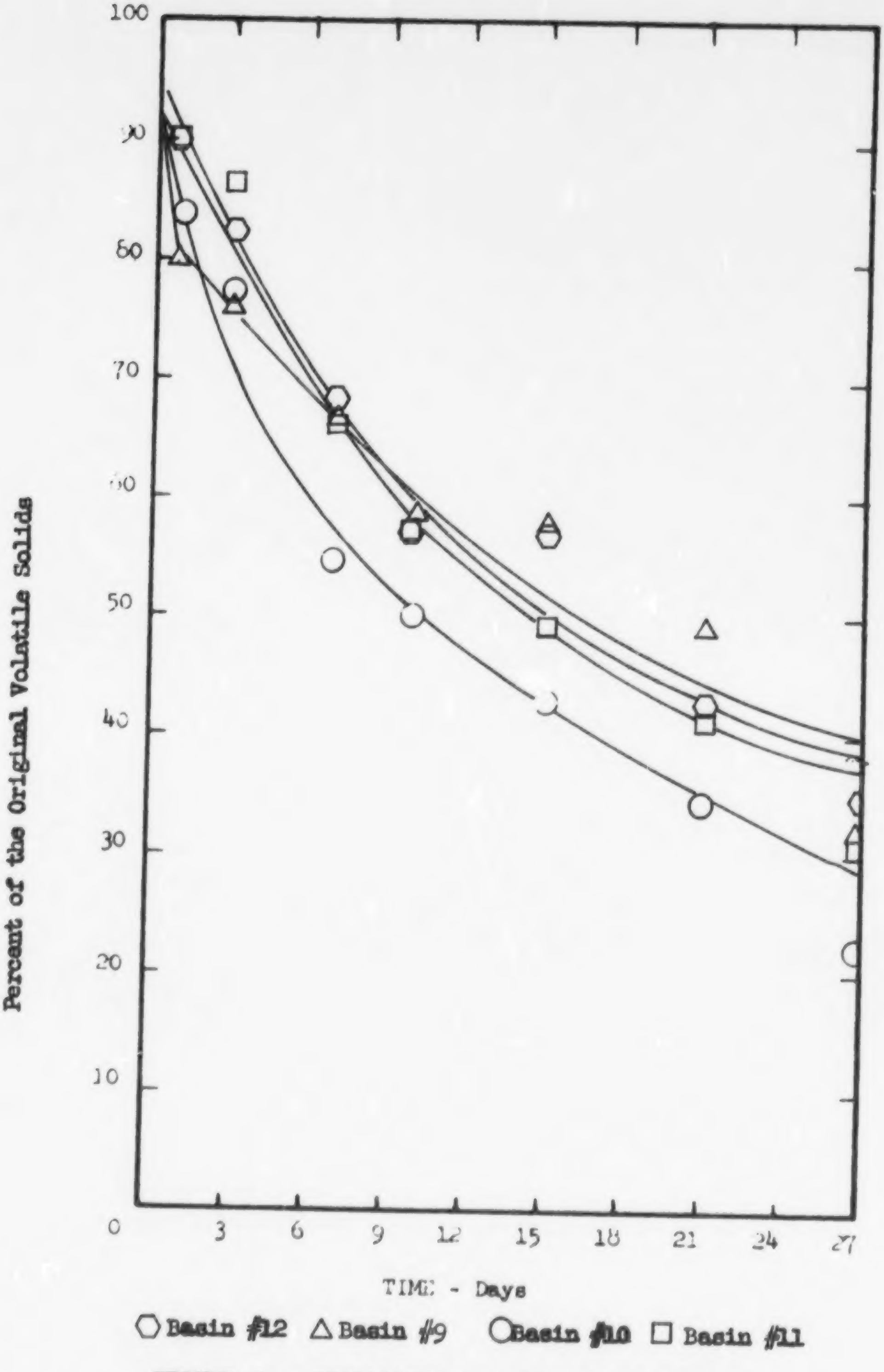


FIGURE 38 - DEGRADATION OF VOLATILE SOLIDS OF GLUCOSE FED SLUDGE

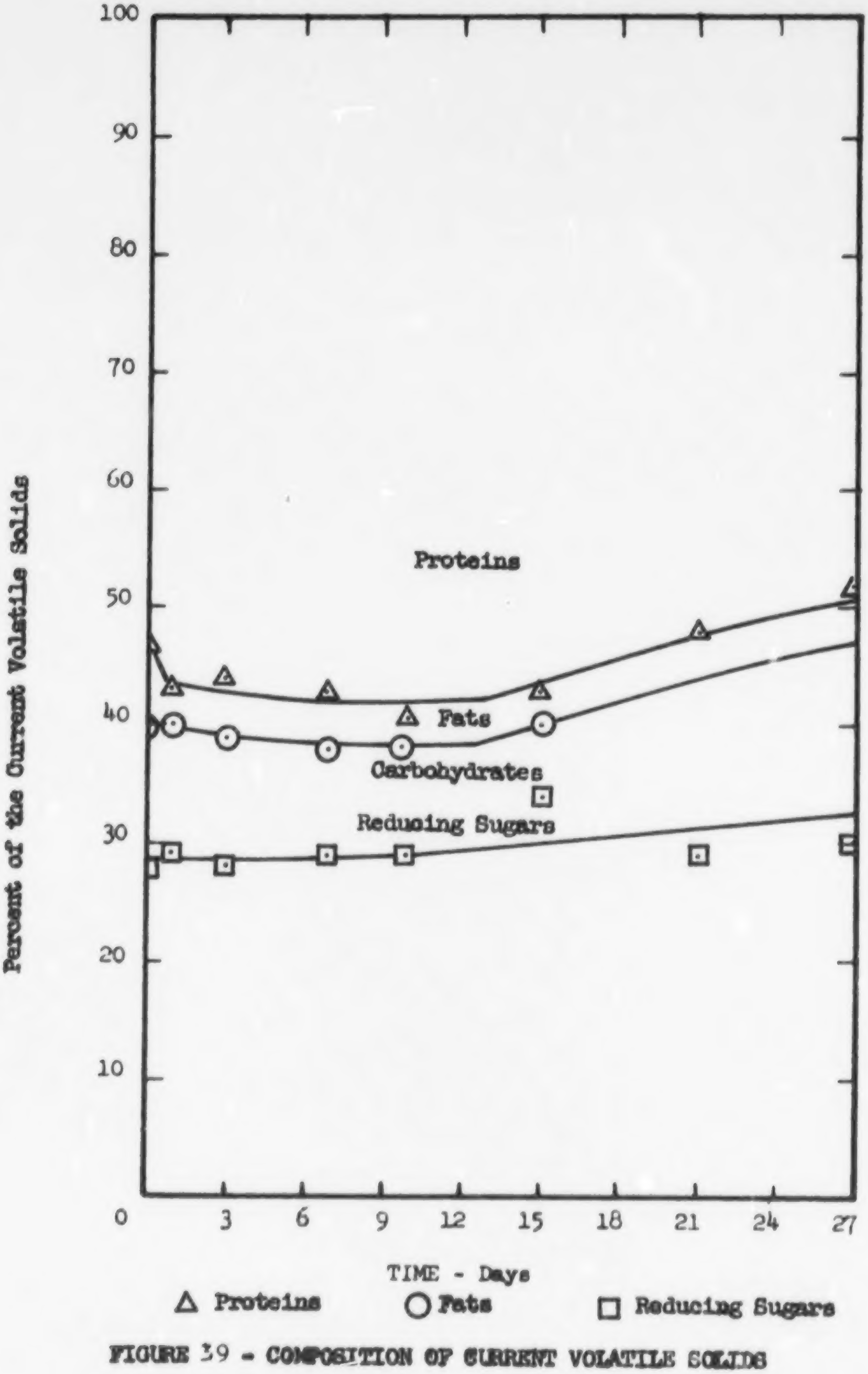


FIGURE 39 - COMPOSITION OF CURRENT VOLATILE SOLIDS

BASIN #9

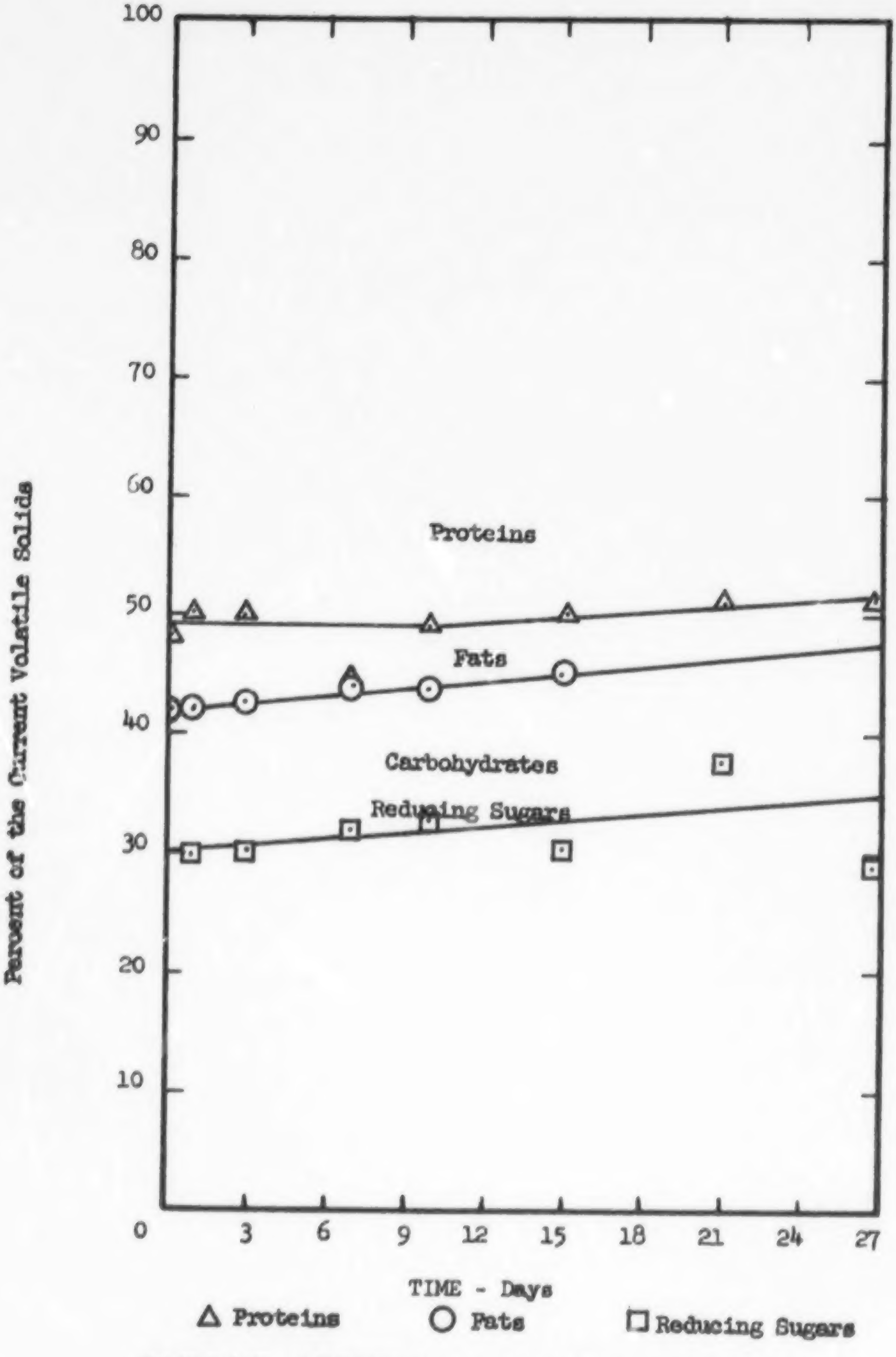


FIGURE 40 - COMPOSITION OF CURRENT VOLATILE SOLIDS

BASIN #10

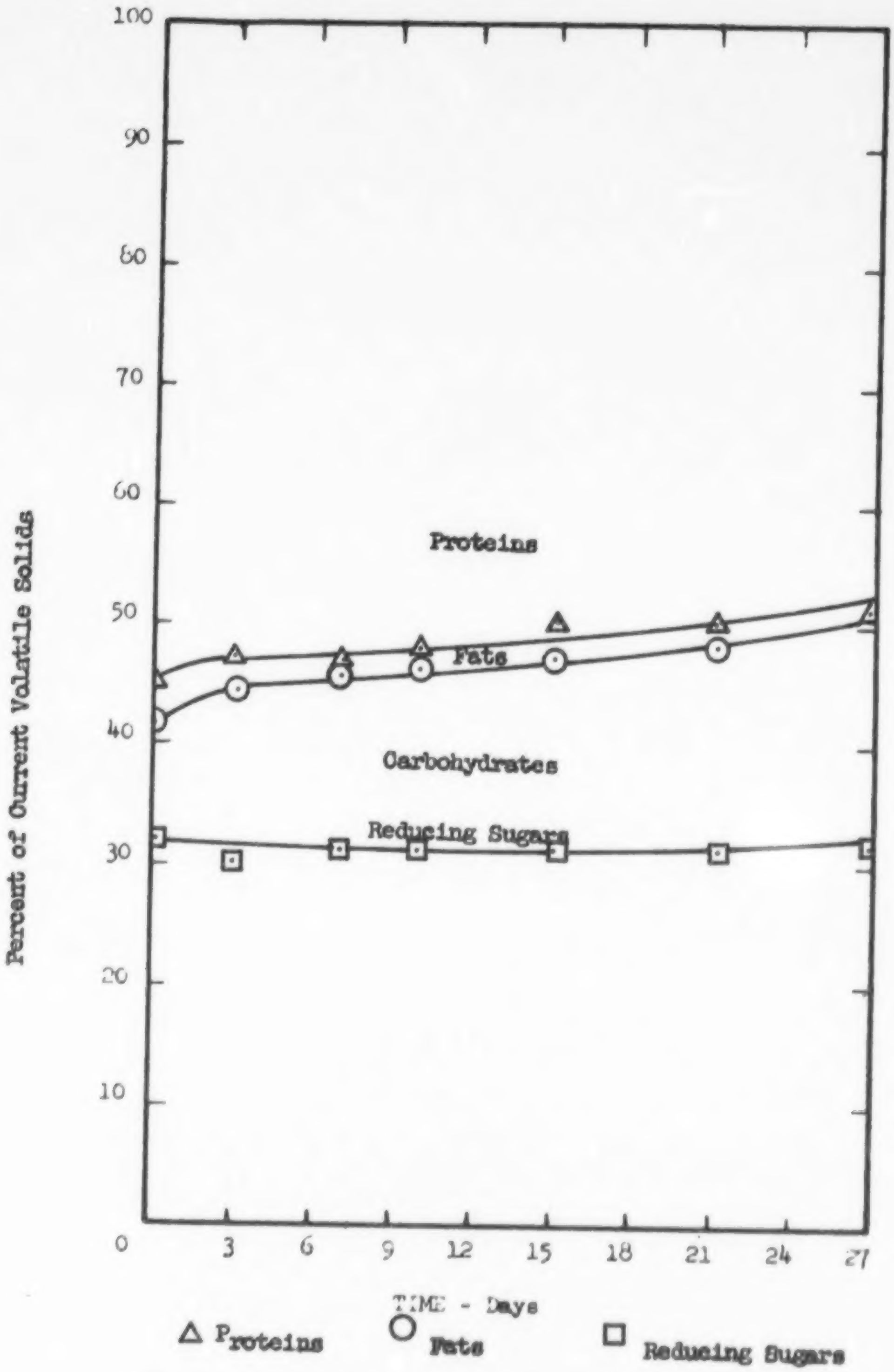


FIGURE 40 - COMPOSITION OF CURRENT VOLATILE SOLIDS

BASIN #11

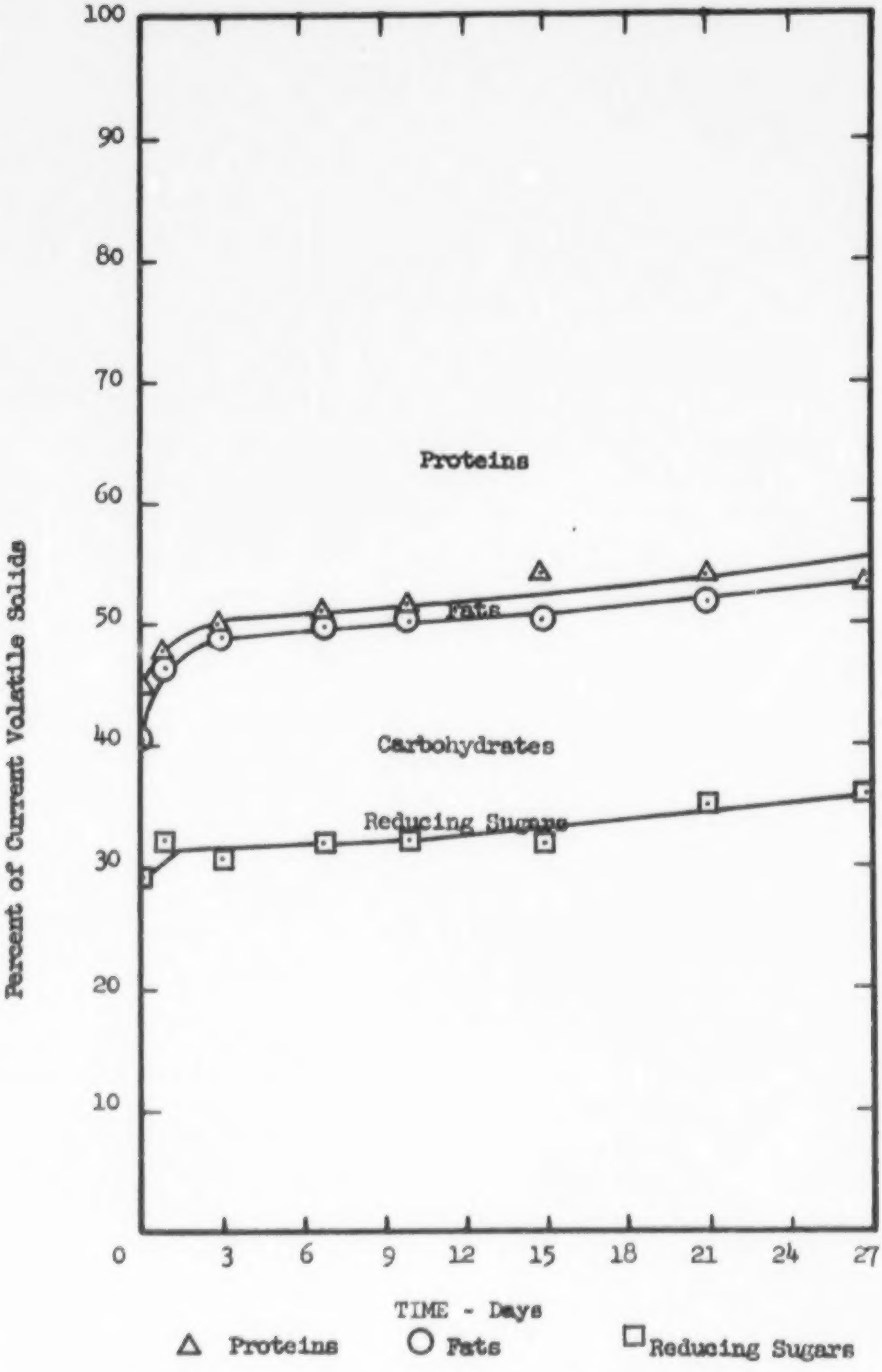


FIGURE 42 - COMPOSITION OF CURRENT VOLATILE SOLIDS

BASIN #12

showed a somewhat larger increase in per cent of volatile solids than the protein decrease due to a decrease in the per cent fats.

It was noted from these curves that the reducing sugars analysis measured approximately the same percentage of the carbohydrate fraction throughout the period of investigation. To show this more clearly, the various relationships of the carbohydrate fractions, taken from Figures 39 to 42 have been summarized in Table XIV. Carbohydrates and reducing sugar values for the original sludge and that on the third and also last day of autolysis have been tabulated. The data from the third day were included to avoid any erroneous conclusions resulting from immediate degradation if it occurred.

The original carbohydrate content of the continuous flow sludges was from 5 to 8 per cent higher than in the acetate-2-C^{14} sludges. This resulted in the carbohydrates representing essentially the same percentage of the initial sludge in all the glucose units, i.e., 40 to 42 per cent. From Table XIV the reducing sugars are seen to measure from 40 to 50 per cent more of the carbohydrates than in the acetate sludges. The reducing sugars continued to measure essentially the same high percentage of the carbohydrates throughout the period of autolysis. This, of course, means that these sludges, unlike the acetate sludges that showed only a slight degradation of carbohydrates, had a large reduction in the per cent of original carbohydrates. Furthermore, the reduction was uniform throughout the carbohydrate fraction as evidenced by the continued high percentage of the carbohydrates measured as reducing sugars.

Summary: All the systems were operated well beyond the point of initial equilibrium. It was noted that after several weeks of equilibrium operation, the carbon balance studies showed that stabilization in the systems approached completeness. Analyses of the sludges at that time showed the per cent carbohydrates in both types of systems to be about the same and that the total sludge in the continuous flow basins was a larger per cent of that in the batch basins than had been the case for the acetate systems.

Micro-analysis of the sludges were made on the 7th and 12th weeks of operation. Over the period there was a drop in the carbon content of the sludge. Also during this period there was a significant increase in the per cent nitrogen in all the sludges. These analyses indicate an increase percentage of proteinaceous material and a decrease in the percentage of carbohydrate material in the sludges.

TABLE XIV

CARBOHYDRATE CONTENT OF GLUCOSE SLUDGES DURING AUTOLYSIS

System	Carbohydrates as per cent of Volatile Solids		Reducing Sugars as per cent of Carbohydrates	
	Current	Original	Current	Original
Basin #9				
Initial Sludge		40		73
Third Day	39	36	74	67
Final Day	46	27	72	49
Basin #10				
Initial Sludge		42		72
Third Day	43	37	72	63
Final Day	48	26	73	45
Basin #11				
Initial Sludge		42		76
Third Day	45	40	71	67
Final Day	51	28	65	42
Basin #12				
Initial Sludge		40		75
Third Day	49	45	66	74
Final Day	53	30	67	50

The carbohydrate data from the synthesis experiment were influenced by the presence of the glucose substrate. The data, however, did indicate that some of the glucose was stored as readily available carbohydrates. The ratio of the proteins and fats synthesized was very comparable to that for the acetate experiments which seemed to indicate the synthesized sludge was comparable to that in the acetate systems.

The protein synthesis in the continuous flow systems again showed possible evidence of carbon dioxide incoporation. Since the substrate was not removed in the batch systems until the fourth hour no effects of such incorporation could be distinguished in these data.

The autolysis data showed very similar degradation patterns for all the systems with from 53 to 59 per cent of the sludge remaining at the end of the period of study. After the initial endogenous respiration, the continuous flow basin sludges were degraded uniformly throughout these components for the duration of the autolysis period. To a somewhat less degree this same degradation pattern occurred in the batch systems. In contrast to the acetate systems, the systems showed a high reducing sugar to carbohydrate ratio. That is to say, the carbohydrates in these sludges yielded 50 per cent more reducing sugars as equivalent glucose per unit weight than did the acetate systems.

E. Glycine-1-C^{14} Systems

The final substrate to be in research was glycine. The glycine represented the protein containing wastes. All the glycine sludges developed similar physical appearances, light brown colored, smooth consistency. The effluents were yellow in color with considerable turbidity. Unlike the activated sludges developed in other substrates, the glycine sludge grew very slowly in both continuous flow and batch systems. The microbial population was also quite different from that of previously developed sludges. While these other sludges had what was considered a "normal" activated sludge population, the glycine fed sludges would not support an animal population. In addition to the periodic inoculum of settled Boston, Mass., sewage, on several occasions well animal populated sludges were introduced in an effort to develop a "normal" activated sludge population. All efforts were futile. It was finally concluded that the glycine fed activated sludge would not support the "normal" sludge animal population.

Carbon Balance: The beginning of the ninth week marked the start of the first carbon balance. One week long balances were conducted to the eleventh week. The summary of the carbon balances for the continuous flow systems #13 and #14 is presented in Table XV. The summary of the carbon balances for the fill and draw systems #15 and #16 is given in Table XVI. Most of the balances closed with less than 5 per cent discrepancy and no closing error exceeded 10 per cent.

The glycine fed sludges grew at a slow pace. After nine weeks, the solids were less than 5,000 mg of volatile matter per basin. Also one of the continuous flow systems contained as much solids as the batch systems. Continuous flow basin #13 had only 2,540 mg of volatile solids by the ninth week. This basin lost a large amount of solids in the effluent as a result of the turbid conditions. From the ninth week of operation, all the glycine fed systems operated at a biological equilibrium as evidenced by the high stabilization. The per cent stabilization was always found to be 78 per cent or higher. At the same time the soluble carbon in the effluent ranged from 12 to 24 per cent of the substrate carbon. This was in sharp contrast to the 95 per cent or greater utilization of substrate observed in the previously studied activated sludges. Thus up to 24 per cent of fed carbon left in soluble form in the effluent need not have been unutilized substrate. As will be shown by the synthesis study, this effluent carbon was for the most part soluble end products excreted to solution from the sludge which was presumably the cause of the yellow color in the effluent. This does not mean that the glycine systems were not at equilibrium for the set conditions of the investigation. There was, however, probably a growth factor lacking in the system which prevented further stabilization of this end product which may have been formaldehyde. This same condition was felt to be the cause of the inability of the system to develop an animal population.

Synthesis: The systems were made radioactive by the introduction of carboxyl labeled glycine (glycine-1-C^{14}) diluted with carrier glycine. The results of the radio assays have been graphically presented in Figures 43 to 46.

During the course of glycine metabolism, nitrate nitrogen was formed due to oxidation of the ammonia liberated. As a result, the glycine fed systems tended to have lower pH values during the metabolism of the substrate. As the glycine was metabolized, the resulting carbon dioxide was evolved as a gas rather than remaining in solution as carbonates. In this study, therefore, mixed liquor assays could be made as a measure of the total volatile solids synthesis.

TABLE XV

SUMMARY OF CARBON BALANCE FOR CONTINUOUS FLOW BASINS - CARBOXYL C^{14} GLYCINE

1 Week in Basin	2 Carbon Removed mg	3 CO_2-C Recovered mg	4 Cell Growth-C mg	5 Effluent Cells-C mg	6 Cells-C Removed during Analysis mg	7 Balance mg-C	8 Closing[1] Per Cent	9 Per Cent Oxidized
System 13 - Continuous Flow								
9								
10	3260	2550	190	485	34	31	9.0	78
11	3020	2520	163	452	50	-185	7.7	84
System 14 - Continuous Flow								
9								
10	3260	2710	-50	254	55	290	8.7	83
11	3380	3000	321	254	75	-270	7.7	89

[1] Calculated on basis of total substrate added.

TABLE XVI

SUMMARY OF CARBON BALANCE FOR FILL AND DRAW BASINS _ CARBOXYL C^{14} GLYCINE

1	2	3	4	5	6	7	8	9
Week in Basin	Carbon Removed mg	CO_2-C Recovered mg	Cell Growth-C mg	Effluent Cells-C mg	Cells-C Removed during Analysis mg	Balance mg-C	Closing[1] Per Cent	Per Cent Oxidized
System 15 - Fill and Draw								
9								
10	3260	2950	-80	117	60	127	10.0	90
11	3450	3530	-295	185	80	50	1.3	100
System 16 - Fill and Draw								
9								
10	3445	2950	170	69	65	190	5.3	85
11	3450	3180	-150	120	93	160	3.1	92

1. Calculated on basis of total substrate added.

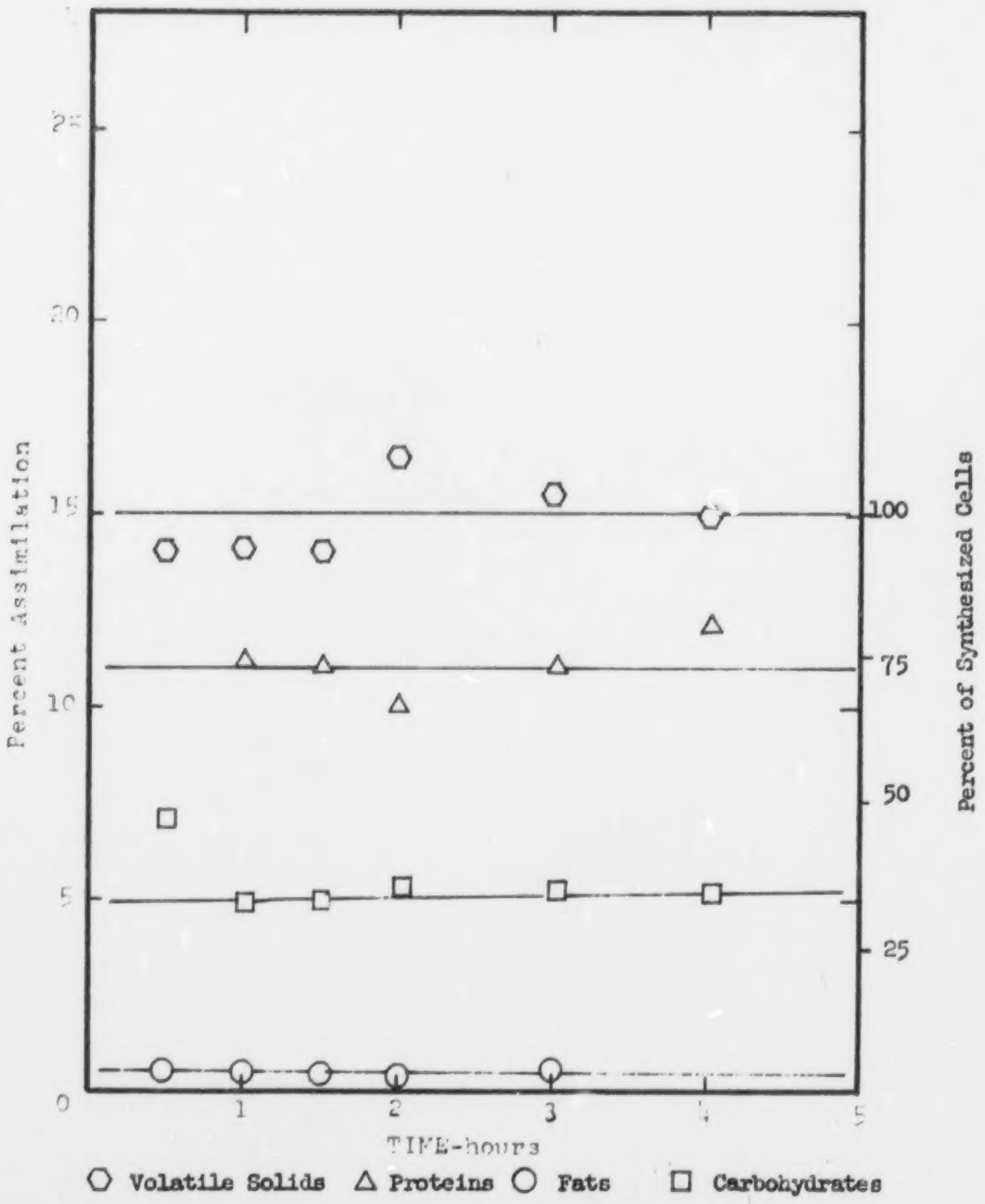


FIGURE 43 - SYNTHESIS OF GLYCINE SLUDGE

BASIN #13

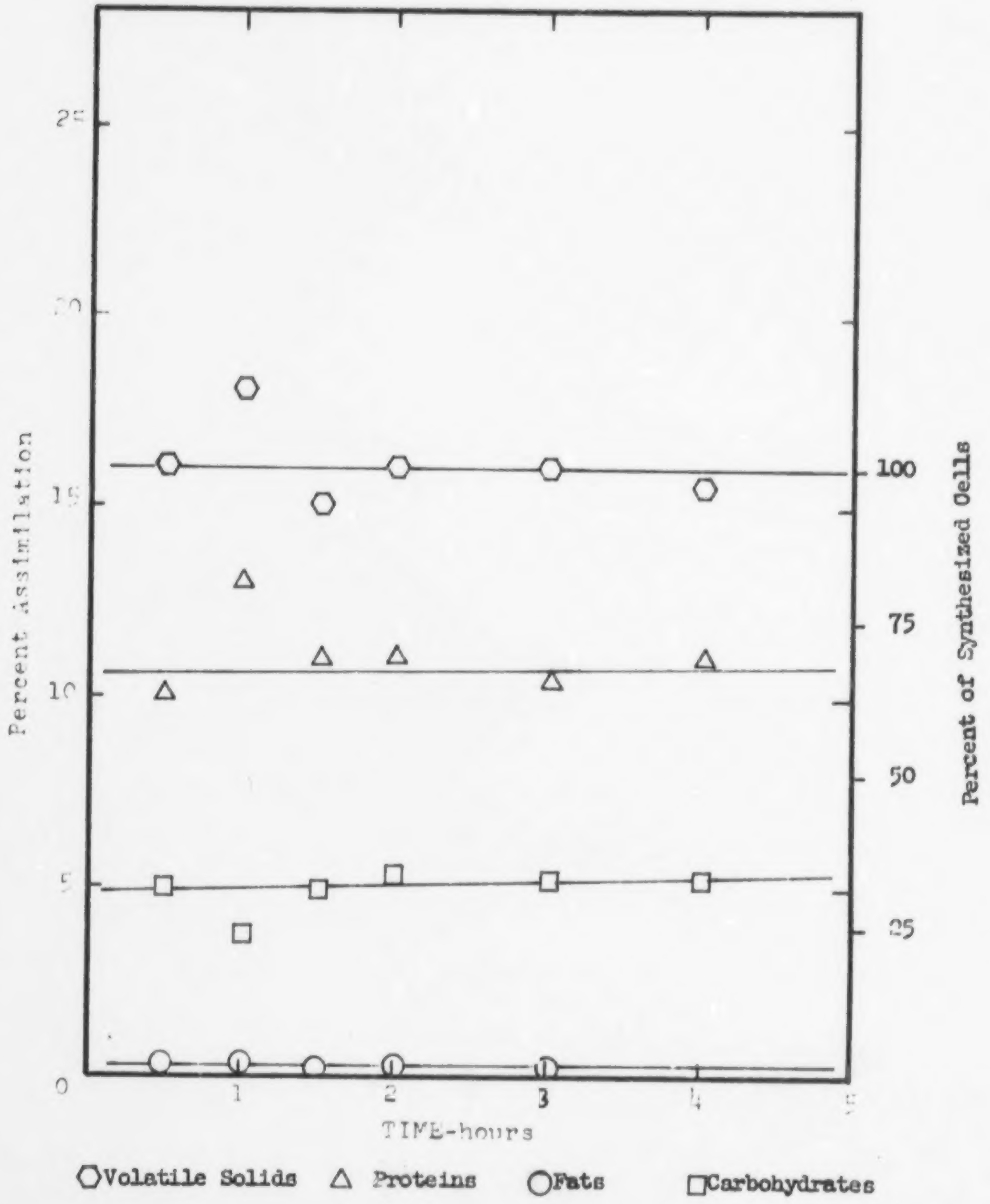


FIGURE 44 - SYNTHESIS OF GLYCINE SLUDGE

BASIN #14

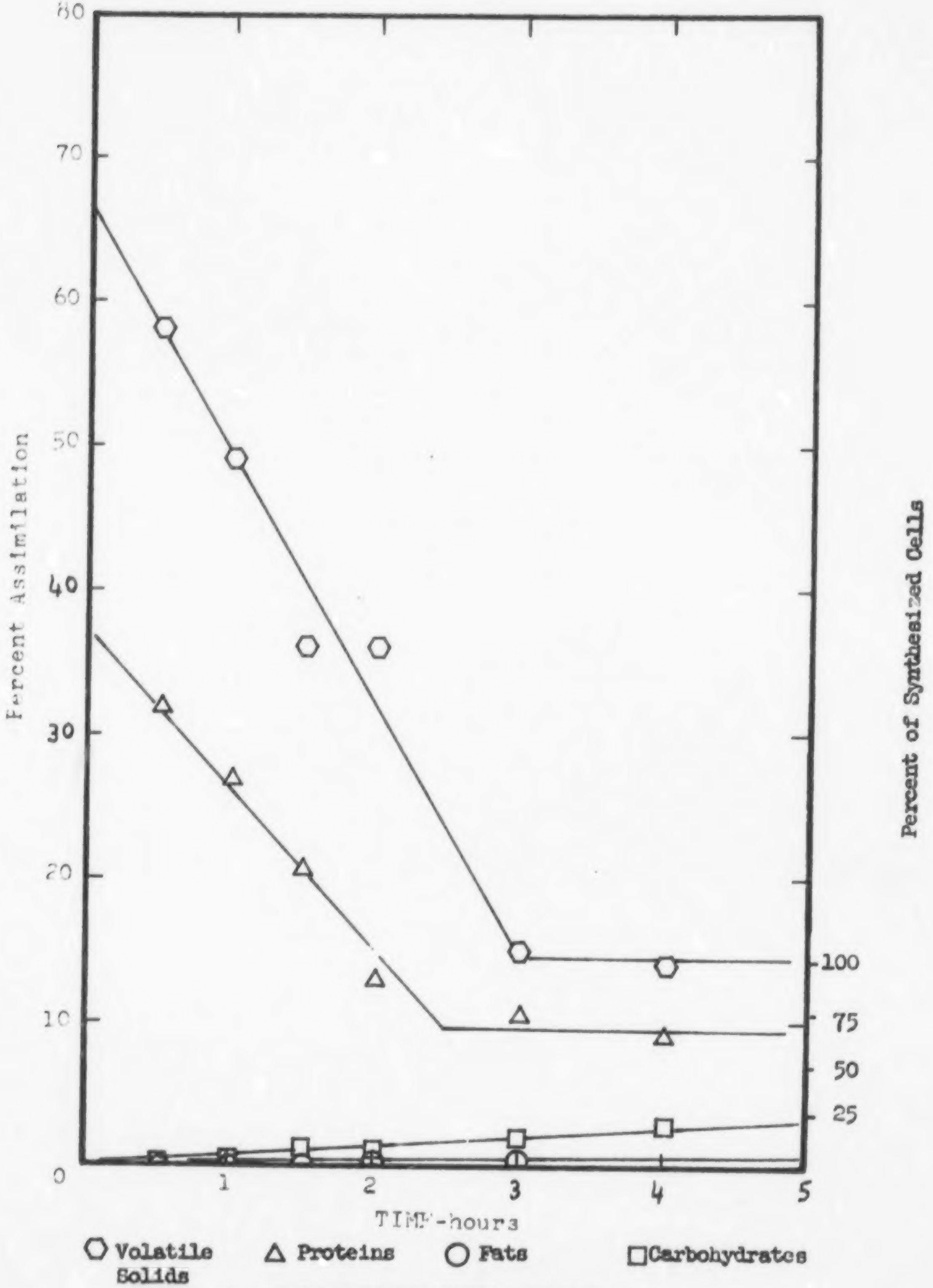


FIGURE 45 - SYNTHESIS OF GLYCINE SLUDGE

BASIN #15

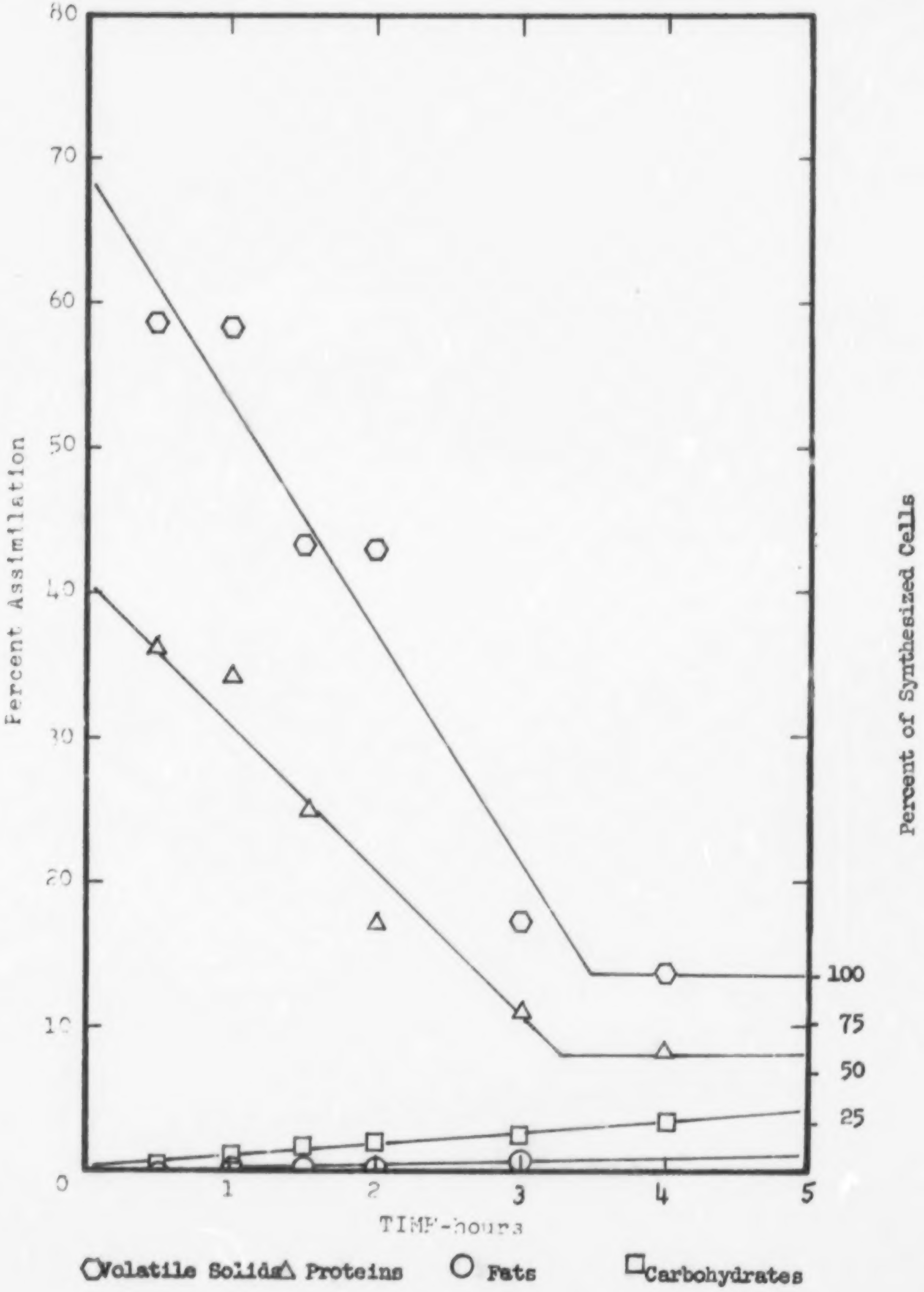


FIGURE 43 - SYNTHESIS OF GLYCINE SLUDGE

BASIN #16

In the continuous flow systems, the glycine was utilized as quickly as it was fed, therefore, no undegraded protein remained in solution. The per cent assimilation or synthesis was the same as the rate of synthesis when both are calculated on the basis of the substrate fed up to a given time. This is shown by the essentially horizontal lines for the assimilation of the various constituents shown in Figures 43 and 44. The curves did not experience a rise due to possible CO_2 incorporation, as had been noted in the previous studies with other substrates, because the pH was sufficiently low to cause all the CO_2 to be evolved as soon as producted. The synthesis in both of the continuous flow systems were in good agreement. The synthesis values for these units are compared below:

Per Cent Synthesis in Glycine Fed Continuous Flow System

Component	Basin #13	Basin #14
Volatile Solids	15.0	16.0
Proteins	11.0	10.6
Carbohydrates	5.0	5.0
Fats	0.6	0.4

For all cellular components the synthesis was within the 1.0 per cent for these basins.

As was expected, the glycine fed to the fill and draw systems showed up as activity in both the volatile solids and protein fractions of the sludge as long as it remained unmetabolized. Consequently, the volatile solids and protein curves in Figure 45 and 46 indicates the glycine which remained in solution for any given time after the last feeding. The sludge in batch Basin #15 took 3 hours to utilize all the glycine. The duplicate batch Basin #16 required between 3 and 4 hours to stabilize the glycine.

It will be noted that the volatile solids curves lie above the protein curves when both essentially measure the same non-utilized glycine. This seeming anomaly was brought about due to the method of calculation of the per cent assimilation from the specific activity. In the case of the volatile solids, all the glycine present was measured in the radio assay and considered to be volatile solids. The volatile solids had a carbon content of 50 per cent while the glycine had but 32 per cent carbon. Thus, according to a sample calculation, the glycine measured as volatile would indicate 50/32 = 1.56 times as much assimilation as if the per cent assimilation was calculated on the basis of a glycine component. Similar error enters the protein calculation for here the protein fraction was considered to have about 50-48/10.32 = 6.3 per cent between the calculated volatile solids and proteins. The difference between the calculated values was greater than this probably due to the fact that the volatile solids measurement measured the total glycine while the protein fraction lost a portion of the glycine during fractionation.

Once the glycine was all metabolized, the synthesis values leveled off. The total measured synthesis for the batch units was about the same as for the continuous flow systems. Actually the batch systems had somewhat higher synthesis rates than indicated from the radio assay data. In these fill and draw systems, the total solids level was much lower than in the batch systems previously studied. Here, the carbon in the feed, which was assimilated into new cells, was an appreciable per cent of the sludge originally in the basin. For example, in Basin #15 the total MLSS was 2,470 mg/l or 3,700 mg/basin of which approximately 1,800 mg was carbon. The 1.0 gm COD of glycine which was fed to the system contained about 400 mg carbon. Of this carbon, perhaps 15 per cent went to synthesis of new cells, or 60 mg carbon. Since the new cells were not considered in the calculation of per cent assimilation, although this carbon diluted the original carbon in the sludge, the measured value was low. In this case, the calculated value was about 60/1800 = 3.3 per cent low.

The composition of the synthesized sludge for the glycine feed systems is given in the right hand margin of the synthesis curves (Figures 43 to 46). For comparison, these figures are given below:

Per Cent Composition of Sludges Synthesized from Glycine

Component	Activated Sludge System			
	#13	#14	#15	#16
Volatile Solids	100	100	100	100
Proteins	74	67	65	60
Carbohydrates	34	33	22	25
Fats	3	2	5	6
Sum of Fractions	111	102	92	91

In contrast to the results obtained in the acetate-2-C^{14} experiment, the composition of the synthesized cells agrees fairly well with the values for new cells given in the literature. In these sludges, the amount of inert material was much less than in the acetate units, therefore, the error introduced due to consideration of the assayed carbohydrate fraction as representative of the actual carbohydrates present in the sludge, was greatly reduced. Thus the measured synthesis in this experiment more closely express^es the true sludge synthesis.

The synthesis of the protein in the glycine-1-C^{14} systems is shown in Figure 47. This figure shows that the duplicate systems were very similar in their synthesis pattern. The continuous flow systems had the same average synthesis or assimilation. The batch systems showed only one per cent difference. The spread of the protein data was only four per cent from a high of eleven per cent to a low of eight per cent. As mentioned above, when the batch protein synthesis was raised about 3.5 per cent as a result of assimilation during the period of synthesis, the batch basins gave an equal if not higher synthesis than did the continuous flow systems. Also the spread of the protein data would be but one per cent, between eleven and twelve per cent, of the utilized carbon.

Autolysis: The glycine fed activated sludge which remained in the basins after the synthesis phase of the synthesis experiment was used to obtain information on the degradation rates of the various cell components.

To present the per cent composition of the sludge it was first necessary to correct the protein data for the apparent protein resulting from the carbon and nitrogen content of the effluent. Since the average stabilization for all the systems in the previous investigations was 96 per cent, it was assumed that a maximum of 4 per cent of the substrate was not utilized, the rest was considered to be by-products of metabolism. Regardless of the exact nature of the proteinaceous material in the effluent, it must all be subtracted from the initial protein value. It seemed reasonable that the end products were utilized slowly as the metabolic pathway was able to reverse itself. For other values then, it was considered that the carbon in the effluent was removed in direct proportion to the time of autolysis over the first ten days of autolysis. The ten day figure was chosen because of a definite decrease in the rate of degradation which occurred at that time. The correction for each basin was calculated on the basis of the average carbon in the effluent for a given basin. The components in each system

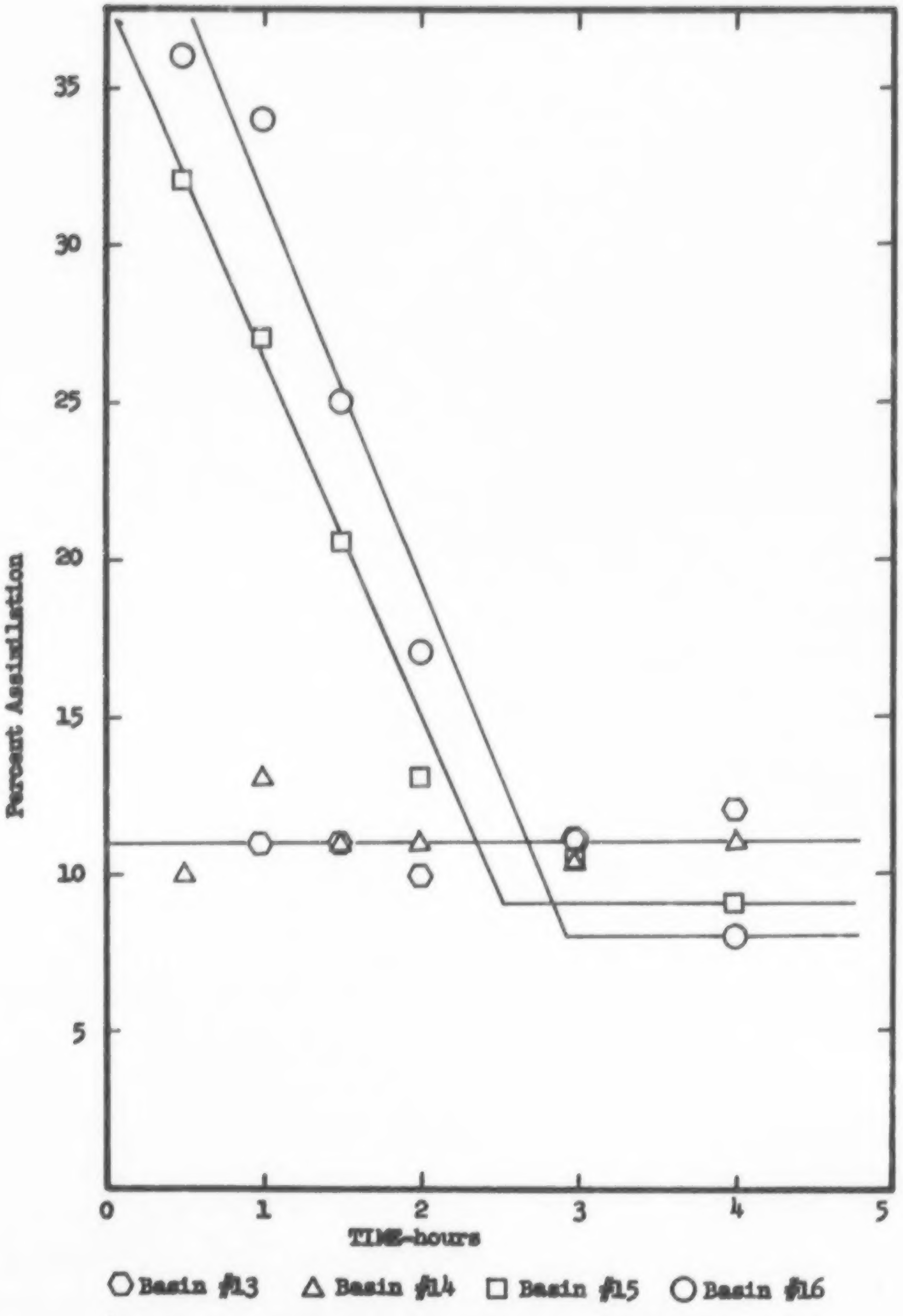


FIGURE 47 - COMPARISON OF PROTEIN SYNTHESIS IN GLYCINE SYSTEMS

as a per cent of the original volatile solids in the systems have been plotted in Figures 48 through 51. The degradation of the volatile solids for each basin is plotted in Figure 52 to show the similarity between duplicate systems.

All the cellular constituents show measurable degradation in all systems. The protein, as in the previous investigation, provides the major source of energy and assimilation. To show the relation of the cell components during autolysis in the glycine grown activated sludges, curves have been drawn of the current composition of the sludge volatile solids, i.e., the sludge composition at any given time during the autolysis phase, from the data in Figures 48 to 51. These graphs for each system are given in Figures 53 to . The ordinates of the graphs represent 100 per cent of the sludge composition at any given time. The upper portion of the graph is the protein fraction. The fats are added to the protein curve rather than the individual points to avoid misleading variations in the fat curve due to poor protein determinations. The lower portion of each ordinate by difference represents the carbohydrate fraction of the sludge. The per cent of the volatile solids measured as reducing sugars has been plotted to readily show the portion of the carbohydrate fraction which these analyses measured.

These curves show that there was, after all, a difference in the degradation process in the systems depending upon the loading schedule. The continuous flow systems showed a definite increase in the carbohydrate portion with increased autolysis time. This change was more marked than in the case of the acetate systems. It was also noted that the reducing sugars showed a slight but steady decrease over the entire period. In contrast, the fill and draw systems showed a more uniform degradation through all the cell components. The current composition was similar to but not quite as constant as that experienced in the autolysis of glucose grown sludges.

To put numerical values to the changes experienced in the protein and carbohydrate components during autolysis, the data from Figures 48 to 56 have been summarized in Table XVII. To avoid any erroneous conclusions resulting from immediate degradation, data from third day were included together with that from the initial and final analysis of the activated sludge.

The percentages of the components given in the Table show how it was possible to have the summation of the material removed from the components, protein fats and reducing sugars, exceed the reduction in the total volatile solids.

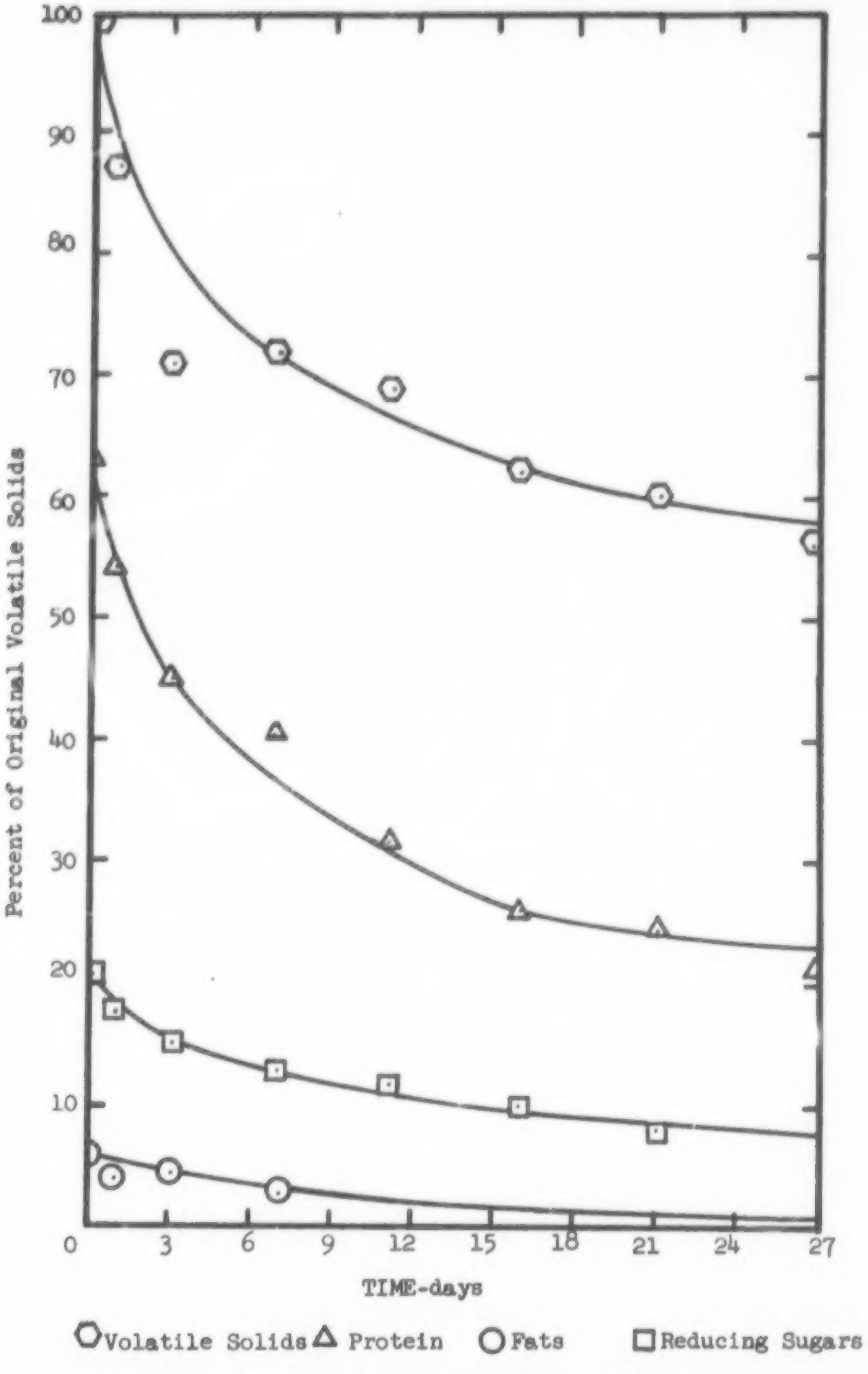


FIGURE 48 - COMPONENTS AS PERCENT OF ORIGINAL VOLATILE SOLIDS

BASIN #13

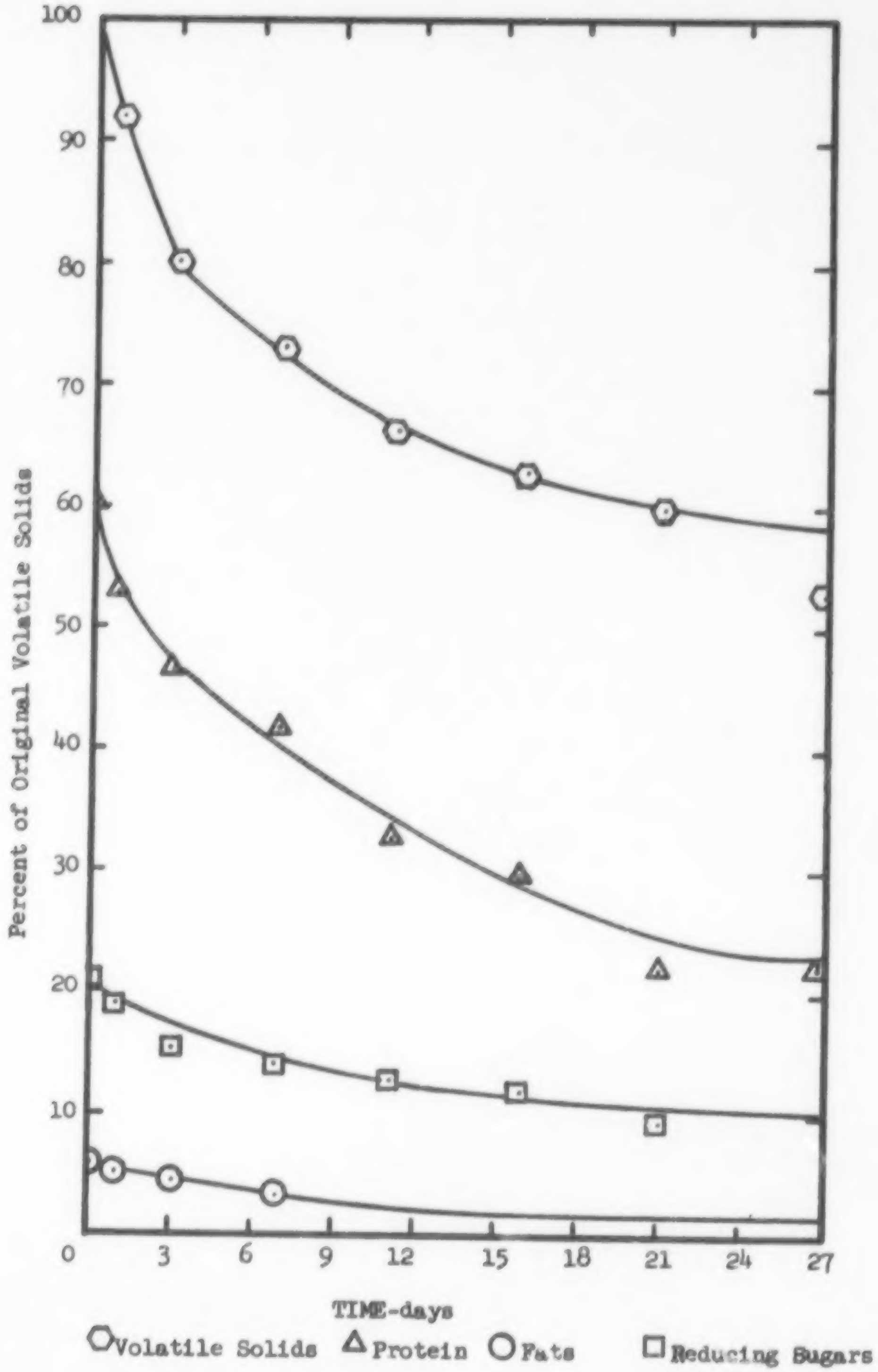


FIGURE 49 - COMPONENTS AS PERCENT OF ORIGINAL VOLATILE SOLIDS

BASIN #14

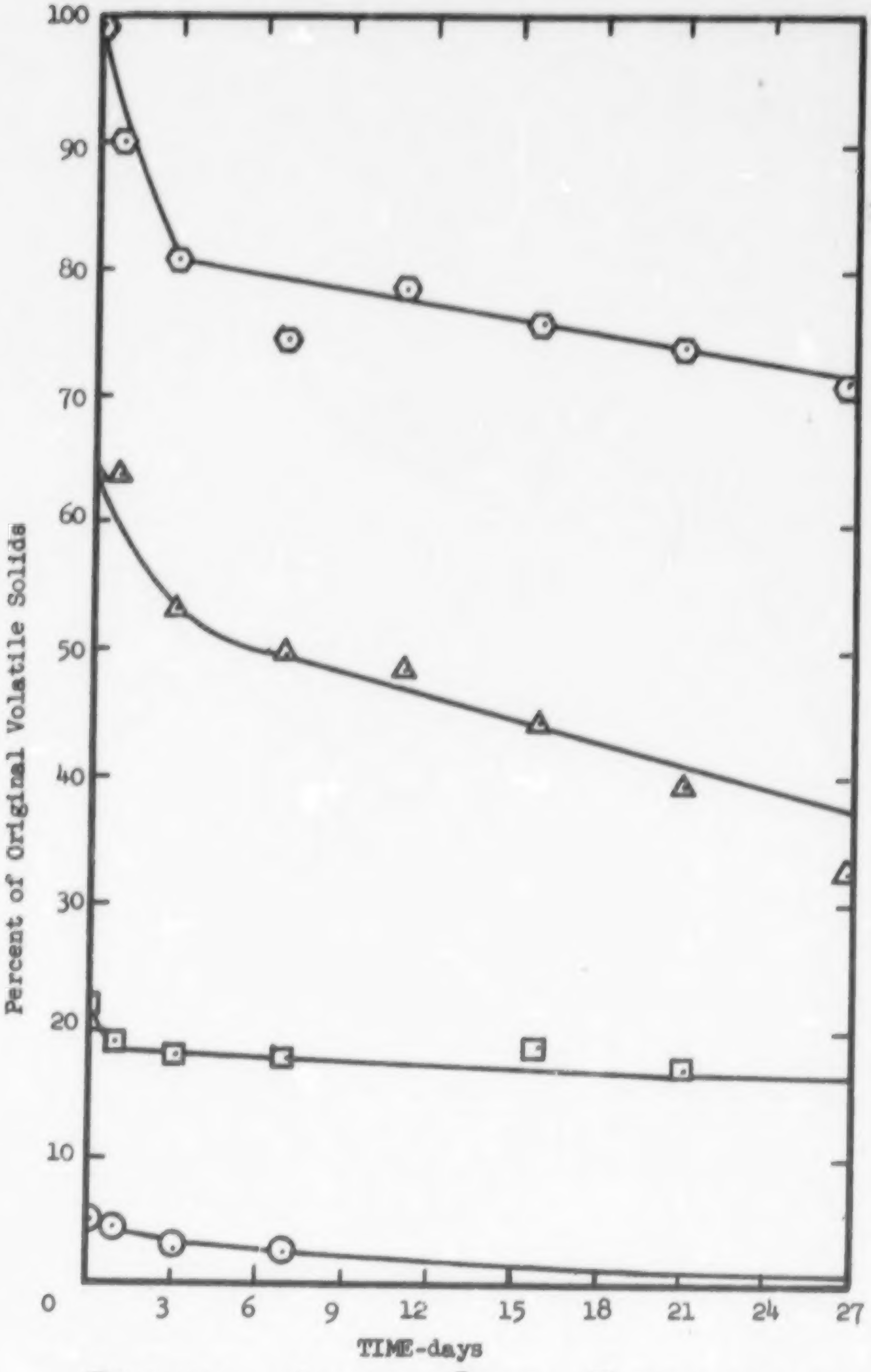


FIGURE 50 - COMPONENTS ~~AS~~ OF PERCENT OF ORIGINAL VOLATILE SOLIDS

BASIN #15

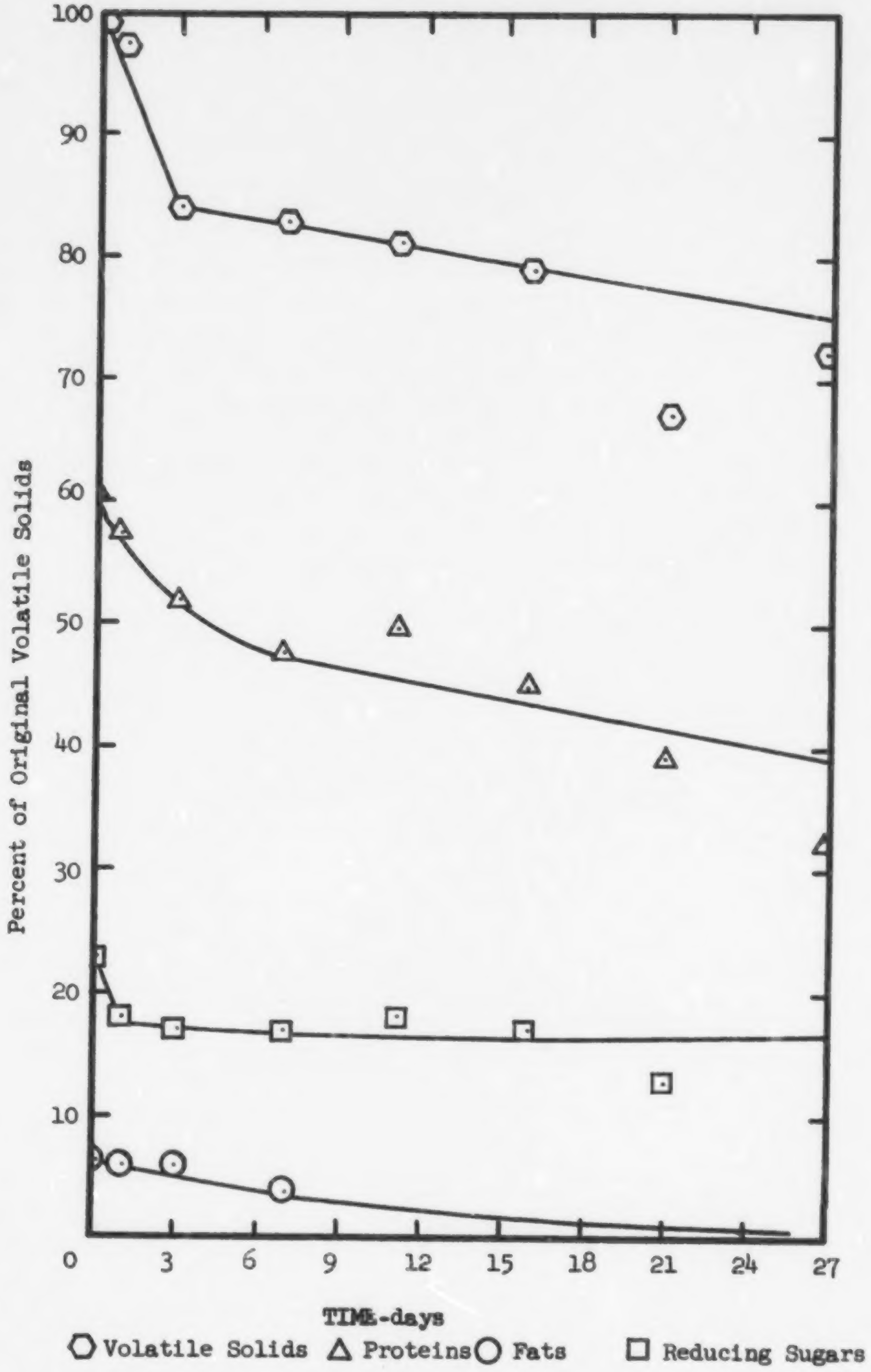


FIGURE 51 - COMPONENTS AS PERCENT OF ORIGINAL VOLATILE SOLIDS

BASIN #16

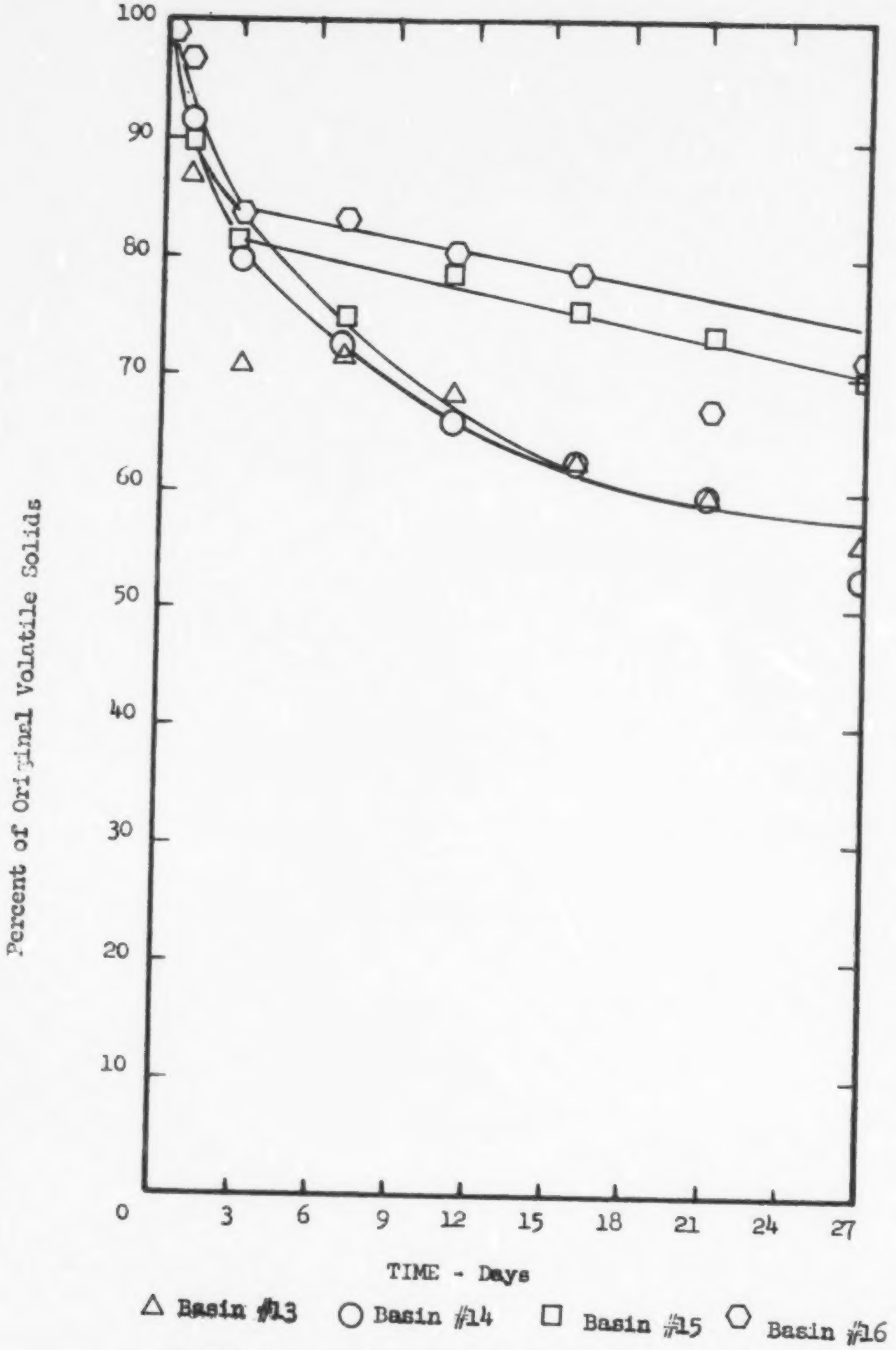


FIGURE 52 - DEGRADATION OF VOLATILE SOLIDS OF GLYCINE FED SYSTEMS

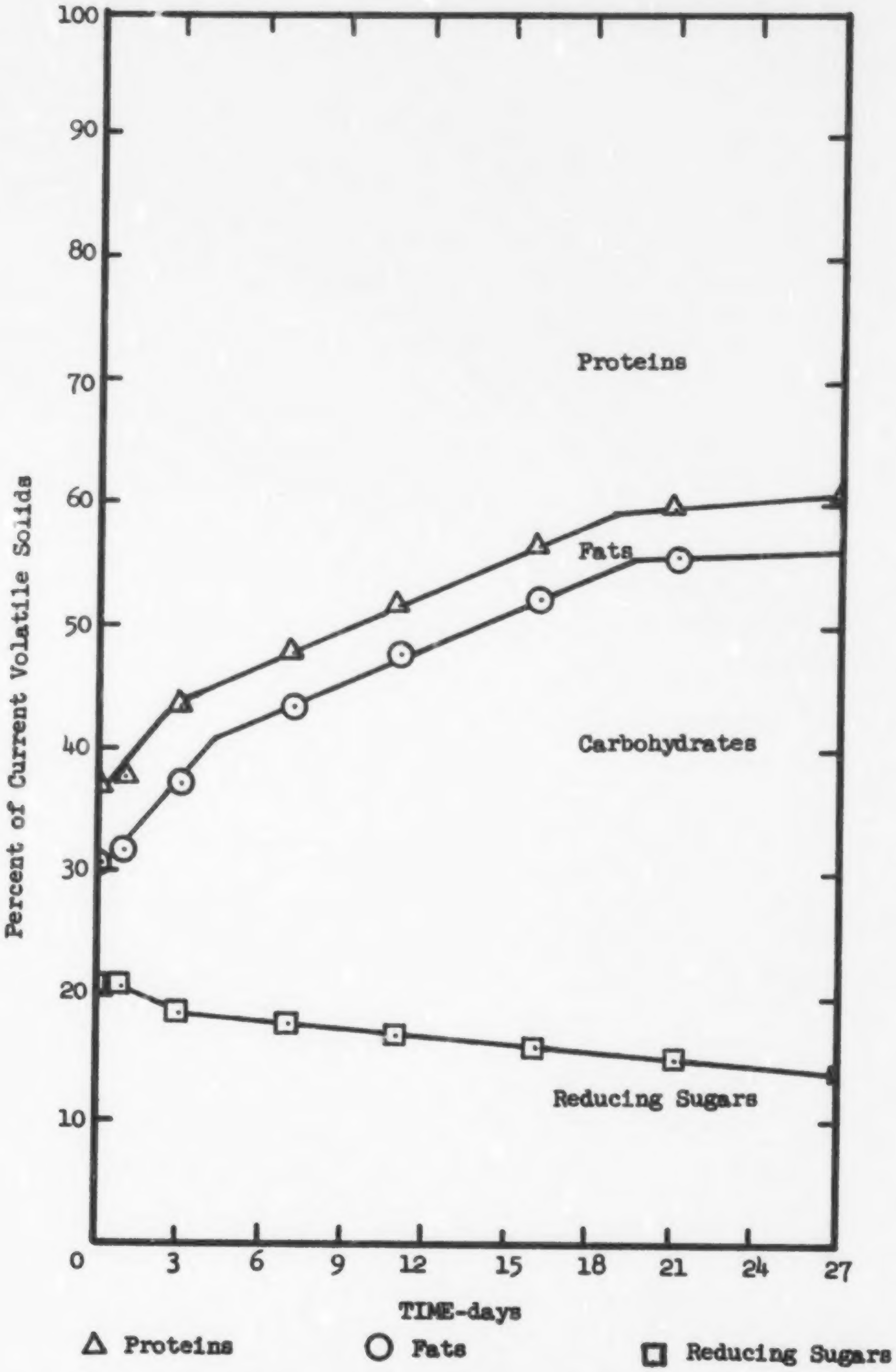


FIGURE 53 - COMPOSITION OF CURRENT VOLATILE SOLIDS
BASIN #13

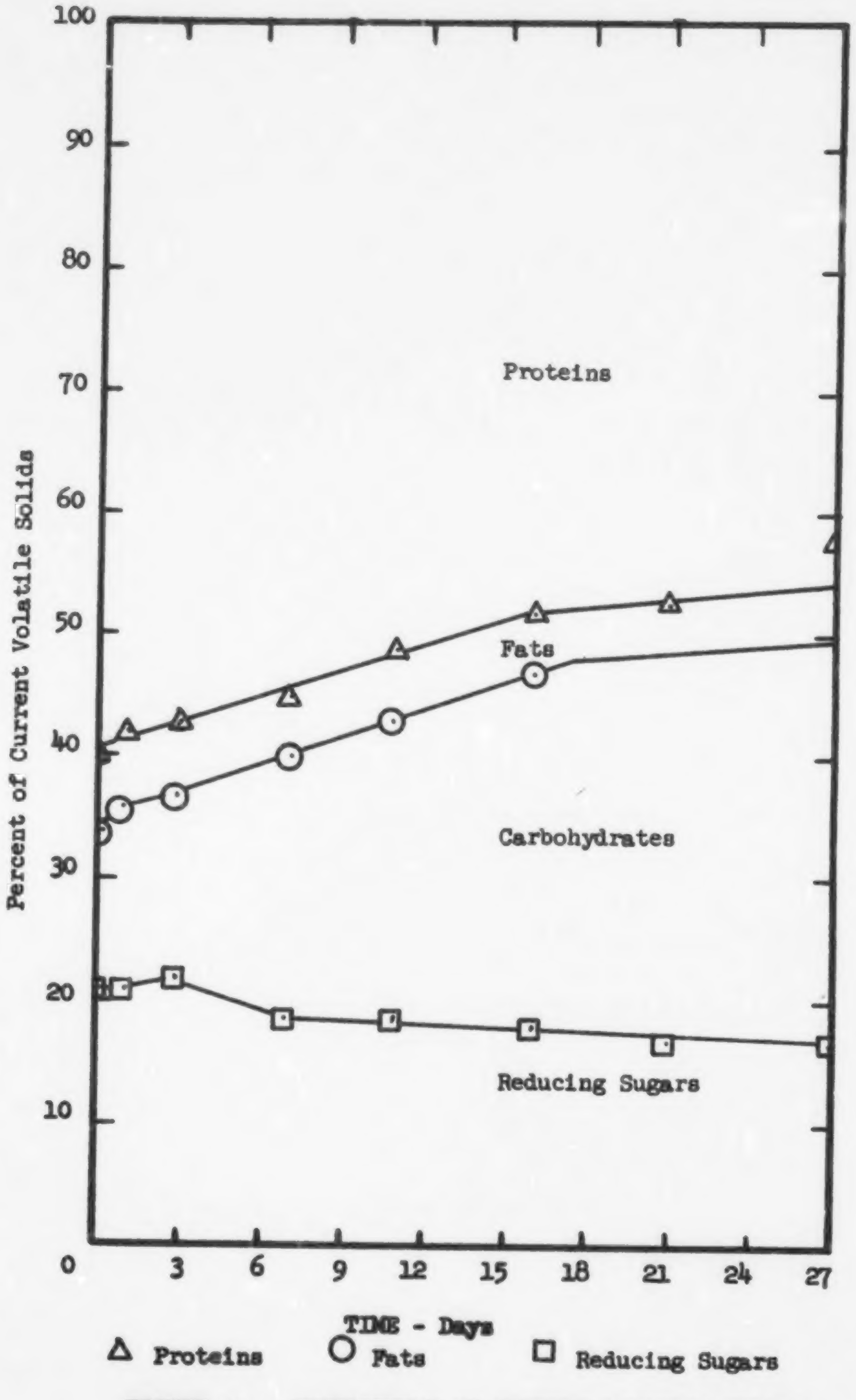


FIGURE 54 - COMPOSITION OF CURRENT VOLATILE SOLIDS

BASIN #14

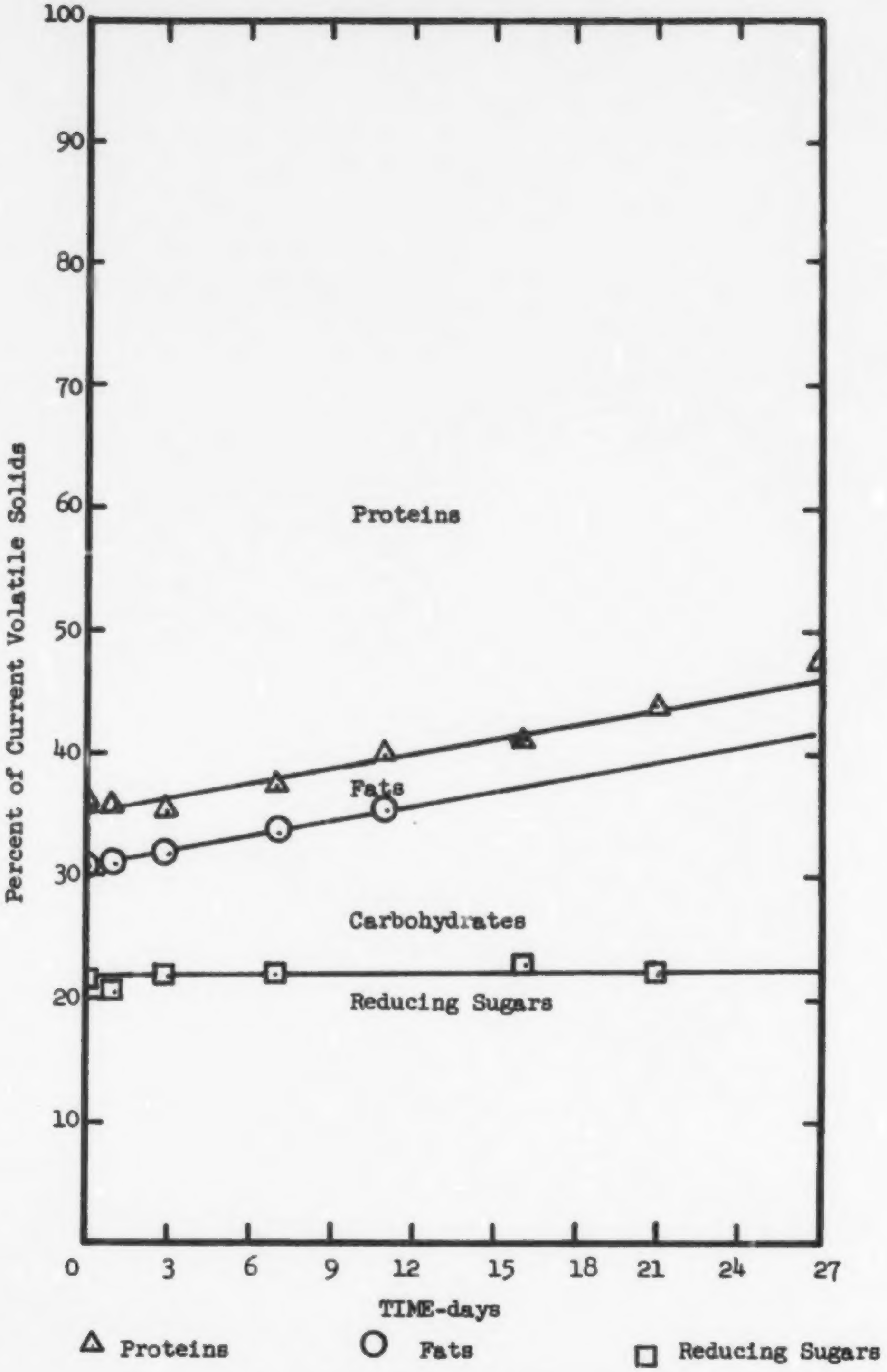


FIGURE 55 - COMPOSITION OF CURRENT VOLATILE SOLIDS

BASIN #15

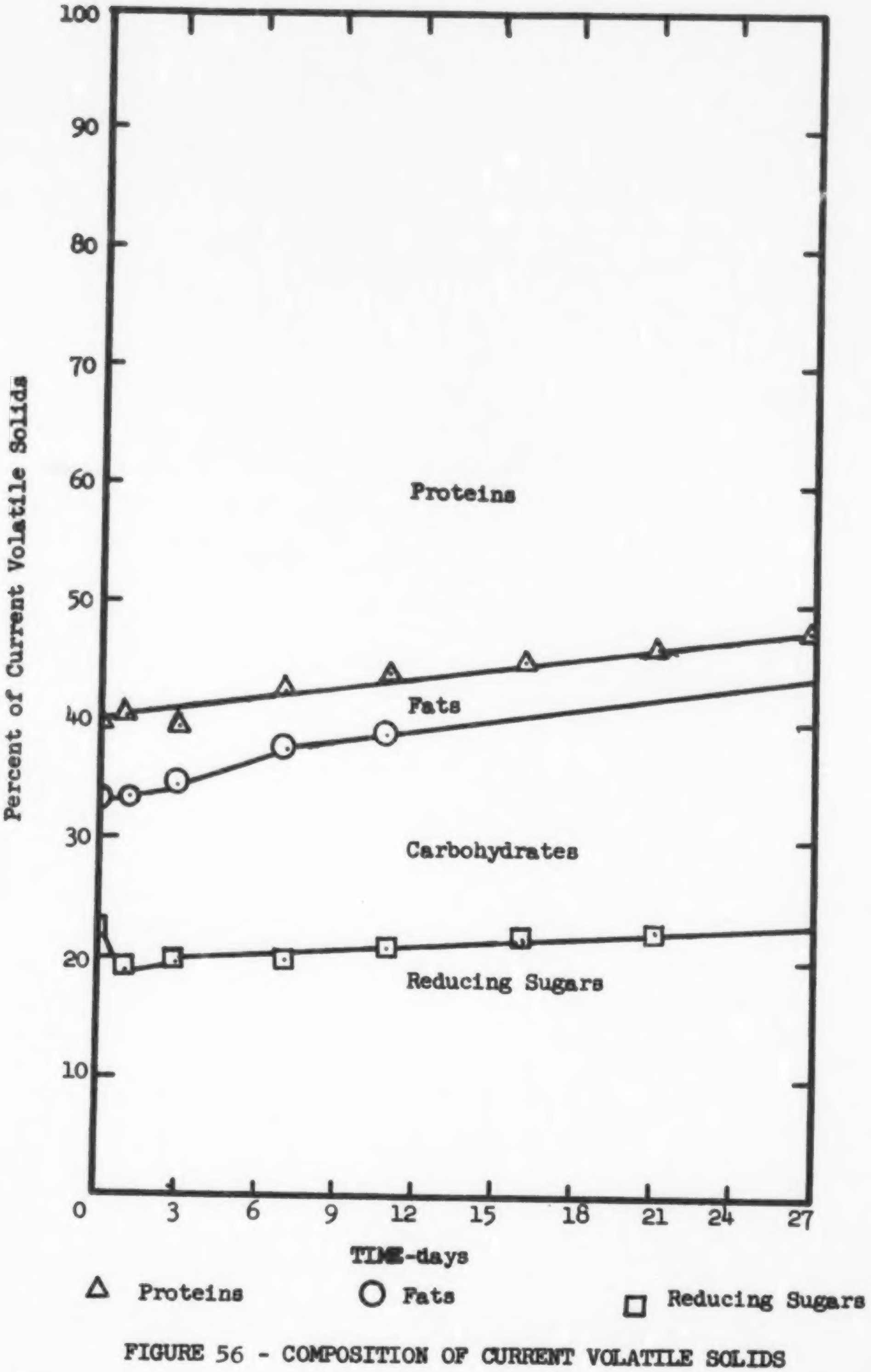


FIGURE 56 - COMPOSITION OF CURRENT VOLATILE SOLIDS

BASIN #16

TABLE XVII

PROTEIN AND CARBOHYDRATE CONTENT OF GLYCINE SLUDGES DURING AUTOLYSIS

System	Protein as Percent of Volatile Solids		Carbohydrates as Percent of Volatile Solids		Reducing Sugars as Percent of Volatile Solids	
	Current	Original	Current	Original	Current	Original
Basin #13						
Initial Sludge	-	63	-	31	-	68
Third Day	56	45	37	30	52	50
Final Day	39	23	57	33	23	25
Basin #14						
Initial Sludge	-	60	-	34	-	62
Third Day	57	46	37	30	59	52
Final Day	46	22	50	29	34	29
Basin #15						
Initial Sludge	-	64	-	31	-	70
Third Day	63	53	32	26	68	57
Final Day	54	37	42	30	52	50
Basin #16						
Initial Sludge	-	60	-	33	-	68
Third Day	59	52	35	28	57	48
Final Day	52	39	44	33	52	52

The difference was evidently made up by an increase in the amount of polysaccharides which yield a lower reducing sugar content than the protoplasm carbohydrates.

It was evident from Table XVII that the batch systems did not experience as large an increase in the non-reducing sugar fraction of the carbohydrates. Here the reducing sugars decreased by only 18 per cent of the total carbohydrates. This lower percentage of increased carbohydrates, however, was a slightly higher proportion of the protein removed than found in the other systems. This condition arose as a result of the hampered degradation which occurred in these batch systems.

Summary: The carbon balance showed the glycine systems to be operating at equilibrium. Effluent from all systems had a high soluble carbon content. This material was shown to be an end product of the glycine metabolism. It was accumulated presumably as the result of a growth factor deficiency. This condition did not, however, hamper the activated process which had a high utilization efficiency. The batch systems showed little growth during the last balance period.

The organic nitrogen content of the sludges in both the continuous flow and fill and draw systems showed little change between nine and eleven weeks but remained constant at around 10 per cent which is the average value for cell protoplasm or active mass. This fact indicated that the sludges contained a high proportion of active mass which was reasonable since the sludge concentration in all units was less than 3,000 mg/l.

Because of favorable pH conditions, it was possible to measure the total volatile solids synthesis. The total synthesis agreed very well with the summation of the three cell components. The assimilation resulting in duplicate systems was practically identical. When corrected for the size of the substrate inocculation, the batch system synthesis was in close agreement with that from the fill and draw systems.

The autolysis pattern was different depending on the loading schedule. The batch systems showed a sharp drop in the rate of endogenous respiration after the third day. The continuous flow systems were uninhibited in the autolysis. In all systems, however, the protein reduction accounted for essentially all of the volatile solids loss over the period of study. Although the reducing sugars as equivalent glucose showed high reduction (50 per cent in the flow through systems), the total carbohydrates remained essentially the same during the entire autolysis period of 27 days. This situation, it was concluded, was the result of accumulation during endogenous respiration if carbohydrate materials not measured by the reducing sugar analysis

F. Sludge Turnover Time

The batch fed activated sludge unit in this study was fed once daily with one liter of fresh substrate while the continuous flow unit was fed uniformly with 1.7 liters of fresh substrate per day. The organic concentration in the continuous flow system was adjusted so that the same quantity of organic matter was fed in both systems over any 24 hour period. The initial organic substrate consisted of 1 gm of acetic acid as sodium acetate and was buffered at pH 6.5 with 3 gm potassium phosphate, monobasic. The monobasic phosphate buffer was used to cause all of the carbon dioxide to be stripped from the activated sludge mixture.

The activated sludge units were allowed to grow up for six weeks prior to their use. In the continuous flow unit with 6500 mg/l volatile solids 81 per cent of the carbon metabolized appeared as carbon dioxide with the remainder being converted to cellular protoplasm. In both batch fed units 84 per cent of the carbon metabolized appeared as carbon dioxide. The solids levels in the two batch fed units were 4040 mg/l and 4320 mg/l volatile solids. For practical purposes there was no significant difference in the metabolism by either the batch fed system or the continuous flow system.

Counting procedures indicated that for the initial study, it would be possible to use 18 μc of acetate-1-C^{14} in each unit along with the regular daily feeding of 1 gm acetate. It was decided that the tracer would be added for one period and that daily analyses would be made of the carbon dioxide recovered over a period of nine days to determine at what rate the carbon was processed through the system and to determine what recoveries could be expected.

Efforts to make a complete carbon balance were not successful in the initial run. The reproducibility of replicate samples of the daily carbon dioxide production was not sufficiently accurate to make quantitative balances. When sufficient carbon dioxide was passed through the traps over the 8 day period, there were no problems with reproducibility or accuracy of the data. It appeared that less sodium hydroxide solution should have been used with the daily samples in order to increase the carbonate concentration to a level which will yield satisfactory accuracy.

Although it was not possible to determine the absolute counts in the carbon dioxide traps for each day, it was possible to obtain the specific activity. The specific activity is a relative measure of the radiocarbon released and can be used to determine in part what was happening in the system. The rate of change in the release of radiocarbon over the 9-day period indicated that 98 per cent of the radiocarbon which would be released had been trapped. Considering the relative rates of carbon dioxide release the following table shows the per cent of total carbon dioxide release for each type of system as the average of two units.

TABLE XVIII

Daily Release of Carbon Dioxide as Per Cent of Total Carbon Dioxide Formed

Day	Continuous Fed	Batch Fed
1	80	81
2	7.2	6.8
3	3.2	3.2
4	2.2	2.2
5	1.7	1.7
6	1.3	1.3
7	1.1	1.1
8	0.90	0.82
9	0.80	0.86

These data indicate that when an activated sludge system is fed a readily utilizable substrate such as acetate, that most of the carbon will appear as carbon dioxide in the first day. After the first day the carbon dioxide release falls off quite rapidly. Theoretical concepts indicate that the carbon dioxide released on Day 1 should come from the synthesis of new cells and the breakdown of a portion of the new cells formed. After Day 1, all of the carbon dioxide should come from cellular breakdown. From these data the turnover time of the sludge is about 7 days in both systems.

DISCUSSION

A. Accumulation

The long-term carbon balance on sodium acetate sludges show that the volatile solids continued to increase during a prolonged period of operation. For the loading employed of approximately 60 lb ultimate B.O.D. per 1,000 cu. ft. aeration capacity per day, the continuous flow systems showed a volatile suspended solid accumulation in the reaction basin of 54 mg/l/day and 46 mg/l/day for the first and second sludges respectively. The total average growth developed in these systems was the same for both systems at 76 mg/l/day. The fill and draw system had an equilibrium suspended volatile solids accumulation of 50 mg/l/day in the reaction basin with a total average growth of 95 mg/l/day. From the results of these long-term Carbon Balance studies, there can be no doubt that a suspended volatile solid material did, in fact, accumulate in these systems.

Data from the carbon balance of the various radioactive substrates has been used to determine average stabilization and accumulation of mixed liquor volatile solids values for these systems operating at equilibrium. These average values for stabilization are given in Table XIX. The average values for sludge accumulation are given in Table XX. The carbon balance data, upon which these values were calculated, were by no means as vast as the information obtained on the long-term carbon balance study. The average values were, therefore, not considered to be as reliable as the values given previously for acetate, but they were sufficient to show the trends to be expected for the different substrates. In those systems where no two consecutive carbon balances showed equilibrium stabilization values, no attempt was made to calculate average values. Also no carbon balance data was considered where during the balance period sludge wasting occured.

Although carbon balances were conducted for shorter periods for the systems used in the radio-tracer studies, the per cent stabilization of substrate, i.e., complete oxidation was shown to range from about 80 to 95 per cent. Since the stabilization was not complete, some accumulation of solids must have occurred in all systems whether they were continuous flow or fill and draw operated. The average accumulation of volatile material which occurred in acetate, glucose, and glycine fed systems is given in Table XX. The range of accumulation was variable from week to week, even so, in the sixteen basin studies the rate of build-up ranged from about 4 to 15 per cent of substrate as new sludge by weight.

These findings are in agreement with the recent report of Kountz and Forney (1) wherein they found 20 to 25 per cent of the weight of the activated sludge produced from the applied substrate remained or was accumulated in the system operating on long-term aeration. They had observed that the influent ultimate oxygen demand had 58 per cent of its weight appearing as new activated sludge in the continuous

TABLE XIX

STABILIZATION OF ACTIVATED SLUDGE SYSTEMS FED VARIOUS SUBSTRATES

Substrate	Percent Stabilization[1]			
	Continuous Flow Systems		Fill and Draw Systems	
Acetate-1-C^{14}	$88_{(3-13)}$	$84_{(5-13)}$	-	-
Acetate-2-C^{14}	$86_{(7-14)}$	$84_{(7-14)}$	-	-
Glucose-U-C^{14}	$89_{(7-12)}$	$83_{(7-12)}$	$92_{(7-12)}$	$85_{(7-12)}$
Glycine-1-C^{14}	$80_{(9-11)}$	$86_{(9-11)}$	$96_{(9-11)}$	$89_{(9-11)}$

TABLE XX

SLUDGE ACCUMULATION OF ACTIVATED SLUDGE SYSTEMS FED VARIOUS SUBSTRATES

Substrate	Sludge Accumulation (mg/1/day)			
	Continuous Flow Systems		Fill and Draw Systems	
Acetate-1-C^{14}	$45_{(3-13)}$	$47_{(5-13)}$	-	-
Acetate-2-C^{14}	$44_{(7-14)}$	$59_{(7-14)}$	-	-
Glucose-U-C^{14}	$44_{(7-12)}$.	$28_{(7-12)}$	$20_{(7-12)}$	$32_{(7-12)}$
Glycine-1-C^{14}	$51_{(9-11)}$	$0_{(9-11)}$	$0_{(9-11)}$	$0_{(9-11)}$

---

[1] The subscripts () give the period of operation of the basins in weeks from which the given value was calculated.

flow system. Therefore, from 11.5 to 14.5 per cent of the substrate B.O.D.$_L$ fed to their system appeared as solids accumulation.

The stabilization as well as the net accumulation for the acetate fed continuous flow systems compares well with the values given above for the long-term carbon balance systems which were also fed sodium acetate. The smaller amount of sludge accumulation which occurred over the measured equilibrium operation period in most of the glucose systems was, except for one continuous flow basin, the result of some periods of negative growth or reduction in solids which took place near the end of the carbon balance phase of these studies. These periods of sludge reduction in the basin were accompanied by relatively higher per cent stabilization. The excessive sludge mass reduction was believed to be the result of some type of sludge adaptation to the metabolism of a portion of the inert material which had previously accumulated. Not considering these unexpected developments of the last few weeks, the accumulation in all these glucose systems was essentially the same as that observed in the acetate systems. It was of importance to note that both fatty acids and carbohydrates gave similar equilibrium accumulation.

The glycine sludge in Basin #13 showed accumulation comparable to that in the acetate systems. The other three glycine fed systems showed no accumulation of sludge over the two weeks tested. On the contrary, the batch systems gave net reductions in sludge mass over this period. The glycine molecule yields less energy on dissimilation than does a corresponding acid or carbohydrate with the same available carbon. The lower available energy in glycine results from the energy loss in removing the amino group. Since less energy is available per carbon atom in this substrate than in the others studied, more substrate must be utilized for energy. As a direct result less synthesis occurs from a given amount of substrate. This phenomenon, although it would explain a smaller amount of synthesis, cannot of itself account for negative accumulation of sludge in the glycine batch systems. A second possible phenomenon is that of adaptation, mentioned above for glucose.

The fact that volatile solids do accumulate in these systems has been shown. Further, according to the radio carbon studies the sludge is continually synthesizing new sludge. These facts verify the theory that at the stationary phase of the growth cycle, the microbial population remains constant as the result of the death rate of cells equalling the growth rate. This is in contrast to the idea that at this phase the organisms only use nutrient for energy source to satisfy their basal metabolisms and process the remainder of the carbon to CO_2. Only by the use of radioactive tracers could this be clearly shown.

B. Inertness of Accumulating Solids

Occurrence of Inert Volatile Solids: It has already been shown that an activated sludge system must, even under so-called complete oxidation operations, produce a continuous increase in sludge mass. It is the purpose of this section to show that the accumulation of sludge in the system after a steady state was reached must have been the result of a build-up of biologically-inert and non-active material. Putting it another way, an equilibrium condition prevailed in such systems. The state of equilibrium has been defined for the purpose of this investigation as that point of operation in the growth cycle wherein the number of active organisms present over a given detention period is a maximum.

Novick has theorized (2) and shown experimentally (3) that the growth in a continuous flow fermenter follows the equation

$$\frac{dN}{dt} = N - \frac{W}{V}N \qquad (1)$$

Where W = nutrient supply (ml/hr)
V = volume (ml)
= mean division rate (div/hr)
N = density of culture (gm/ml)
t = time in hours

The equation states that the rate of accumulation of cells is equal to the number of cells times their division rate minus rate of washout of the cells in the effluent. It is evident that the washing out time should be lower than the maximum growth rate due to the division time of the organism to maintain a positive growth. For any given conditions a population will become established in which the growth rate will equal the wash-out rate. What happens is that the number of cells will increase until the growth equals the wash-out. Thus a steady state population will be developed for a certain food concentration and detention period.

In the case of the activated sludge basins, the sludge is settled from the discharging liquid. In effect the detention period or washout rate approached zero as far as the sludge is concerned. From Novick's equation, as the detention period approaches infinity, the steady state population approaches a maximum value. This is the equilibrium population. Bilford, et al. (5) has shown that an equilibrium population does exist for continuous flow systems. While Forges, et al. (5) has shown the development of an average equilibrium population in a semi-continuous or batch fed system. It follows that if the maximum viable population of organisms exist at the start of the equilibrium phase, all the accumulation of sludge during equilibrium operations must be inactive mass. Also it must be biologically inert or it would be removed due to endogenous respiration.

Evidence has been accumulated in all phases of this investigation that show that there will be a buildup of inert volatile solids in a long-term aeration activated sludge system operating at biological equilibrium. The occurrences of this inert solids accumulation will be independent of the type of substrate whether it be protein, fat or carbohydrate in nature, provided sufficient mineral nutrient is provided.

The inert solids build-up was most clearly observed from the Long-term Carbon Balance Studies. In equilibrium systems grown on 40 lb. COD/1000 ft^3 total basin capacity/day sodium acetate, the sludge weight increased at a relatively constant rate regardless of the type of system. It follows from the basic assumption (that equilibrium systems contain the maximum weight of microorganism that can survive for the given substrate loading) that these volatile solids accumulating during equilibrium were non-active and biologically inert. Under conditions of no intentional sludge wasting (0.6 - 0.7 per cent actual wasting - sludge retention time 142 to 166 days) and no observed sludge adaptation to the inert material, these uniform concentration acetate fed systems showed an average inert volatile solids accumulation as mass of 11.5 per cent of the ultimate B.O.D. removed and 16.9 per cent of the BOD$_5$ removed. The similar conventional system showed 14.5 per cent accumulation of the BOD$_1$ removed and 21.3 per cent of the BOD$_5$ removed.

Composition of Inert Volatile Solids: The inevitable accumulation of organic solids in an activated sludge system operating at equilibrium has been shown in the previous section. It is the purpose of this section to define the chemical composition of the inert fraction in the general terms of the major cell fractions, proteins, fats, carbohydrates.

The qualitative determination that the biologically inert material which accumulated in the activated sludge systems was composed of a portion of all three components was shown in the first autolysis experiment. In this experiment acetate grown sludges, operating at equilibrium, were made radioactive by feeding carboxyl labeled acetate. The specific activity of the sludge components all rose to a maximum value and then declined. This change in specific activity, which was graphically shown for the protein components in Figure 14, was evidenced in all the components, proteins, fats and carbohydrates. Briefly, in a homogeneous protoplasm the synthesized radioactive cell components would mix with the previously formed portion of that component producing an increasing specific activity which would reach a maximum when the substrate was exhausted. As a consequence of this homogeneous condition in the component, the specific activity would remain at this maximum level during degradation. Since this was not the case, there must have been present in the sludge a certain portion of non-active material which did not mix uniformly with the synthesized sludge component. Table XXI, reproduced below, shows that there was a substantial decrease in the specific activity in all the components over 27 days of autolysis. This decrease could only mean that the most recently formed portion of the components was degraded by endogenous respiration more rapidly than the average of the components. Since in the active or viable cells, the synthesized material is homogeneously mixed with the previously synthesized portion, it follows that the drop in specific activity was the result of the non-active mass being less biologically degradable than the active portion. It has thus been shown that there is a build-up of non-active and biologically inert material in the activated sludge systems at equilibrium. Further, this mass is composed in part of all three of the cell components, proteins, fats, and carbohydrates.

TABLE XXI

REDUCTION IN SPECIFIC ACTIVITY DURING AUTOLYSIS OF ACETATE-1-C^{14} SLUDGES

	Percent Activity Remaining After 27 Days					
	Continuous Flow Basin			Batch Basin		
Component	#1	#2	Avg.	#3	#4	Avg.
Proteins	70	81	76½	58	67	62½
Fats	68	71	69½	57	74	65½
Carbohydrates	40	43	41½	27	33	30

Another proof of the predominence of polysaccharide material in the biologically inert material accumulating in the system was obtained from the protein content for the volatile sludge solids in the various equilibrium systems. The volatile organic material in bacterial cells contains an average of 70 per cent protein. Since these studies were conducted with mixed cultures, it is reasonable to assume that the active cells in the activated sludge systems contained about 70 per cent protein. Chemical analyses were made of each sludge just prior to the tracer studies of synthesis. The systems had been operating at equilibrium for several weeks. The protein calculated from these analyses for all sixteen systems is given in Table XXII. The protein content of all the sludges was considerably less than that of bacterial cells. The activated sludge grown on amino acids was expected to have a higher protein content than sludges grown on fatty acids or carbohydrates due to the fact that the substrate was a protein derivative. Since the fat content of the sludges was small, these sludges must have accumulated large quantities of inert polysaccharides for the volatile solids are composed largely of proteins, carbohydrates and fats. Thus it may be concluded that the major components of the biologically inert volatile organic matter that accumulates in an activated sludge system are polysaccharide in nature.

TABLE XXII

Protein as Per Cent Volatile Solids

Substrate	Continuous Flow Systems		Fill and Draw Systems	
Acetate-1-C^{14}	57.0	59.0	43.6	40.6
Acetate-2-C^{14}	59.2	58.6	44.5	46.2
Glucose-u-C^{14}	52.5	52.0	55.0	54.0
Glycine-1-C^{14}	61.0	60.2	64.5	64.0

The above was also supported by the data from the autolysis studies wherein the carbohydrate fraction of the sludge increased with time of autolysis. This increase in the carbohydrates indicates that there was a larger portion of biologically inert material in the carbohydrate fraction than in the other cell fractions. For the protein and fat fractions were shown to be readily degradably during endogenous respiration by the fact that these fractions were reduced to a considerable extent during the course of the experiments. The carbohydrate increase in the composition of the sludge solids during autolysis is summarized in Table XXIII. It should especially be noted that the largest increase in carbohydrate content of the sludge occurred during the autolysis of the glycine sludges. Although the existence of inert polysaccharide material was not manifested in the carbon balance data, as discussed previously, this inert build-up was clearly shown by these autolysis results.

The observation that the inactive mass contains nitrogenous material is of prime sanitary engineering significance. Since the nitrogen is continuously being taken up in biologically inert material it is not available to the system for future synthesis. It must, therefore, be concluded that no activated sludge system can be operated with only sufficient mineral nutrient to make up that lost through the effluent as was proposed by Symons (6). But rather it is essential that nitrogen be supplied to make up for that synthesized into inert material as well. The latter amount represents the absolute minimum nitrogen requirement for normal operation of an activated sludge system operating at equilibrium without sludge wasting. A rough quantitative measure of the minimum nitrogen requirement for the loading of 1 gm C.O.D./basin/day can be obtained from the long-term carbon balance study. The nitrogen and volatile solids accumulation during the long-term carbon balance for both loading schedules is shown in Figure 8. Based upon the percentage of nitrogen present in the sludges and their rates of accumulation, nitrogen requirements have been calculated as shown in Table XXIV. Because of the approximate nature of the evaluation only the average for the two continuous flow systems were used.

It will be noted that the calculated accumulation of nitrogen in the inert sludges was quite similar. The variation, such as it was, was measured from grab samples. Based upon these calculations an activated sludge system loaded at 40 lb of oxidizable organics/1000 cu. ft. of total capacity/day would require from 0.27 to 0.24 lb of nitrogen /10000 cu. ft./day. This means that the maximum ratio of 5-day B.O.D. to nitrogen would run about 150:1 in normal operation.

The composition of the inert volatile solids can be estimated in terms of the three major cell components from the data obtained during the course of this investigation. It has been shown by bacteriologists that a system developed on a nutrient limited substrate is essentially totally active up to the point of equilibrium. The volatile solids in the continuous flow system were about 2500 mg/l at equilibrium.

TABLE XXIII

CHANGE IN CARBOHYDRATE OF ACTIVATED SLUDGE DURING AUTOLYSIS

Substrate	Basin No.	Carbohydrate as percent of Volatile Sludge Solids		Percent Increase over Period of Autolysis
		3 days	27 days	
Sodium Acetate				
Continuous Flow	5	37	53	16
Continuous Flow	6	39	52	13
Fill and Draw	7	47	60	13
Fill and Draw	8	50	57	7
Glucose				
Continuous Flow	9	39	46	7
Continuous Flow	10	43	48	5
Fill and Draw	11	45	51	6
Fill and Draw	12	49	53	4
Glycine[1]				
Continuous Flow	13	37	57	20
Continuous Flow	14	37	50	13
Fill and Draw	15	32	42	10
Fill and Draw	16	35	44	9

1 - Glycine data was corrected for apparent protein.

Not all of these solids were active, however, for during the first week the system operated under a batch loading schedule. During this initial week 1,120 mg of sludge were developed in the basin, i.e., 750 mg/l. Of these initial solids about 40 per cent were active based upon the ratio of sludge concentrations at initial equilibrium for both batch and flow through systems. Subtracting the initially developed inert solids gives an active mass at equilibrium of 2050 mg/l. It is recognized that there is no reliable measure of active mass and that the above figure is therefore open to some question. If the value used for active mass is too high, this would merely result in more inert material. With the value of the active mass determined in this investigation, it is possible to estimate the composition of the inert solids based on the chemical analysis of the sludges at equilibrium.

TABLE XXIV

NITROGEN ACCUMULATION IN ACTIVATED SLUDGE AT EQUILIBRIUM

Accumulation Average	Continuous Flow Basins	Fill and Draw Basin
Volatile Solids mg/basin/day	75	75
Nitrogen percent	8.2	9.0
mg/basin/day	6.1	6.7
lb/1000 cu.ft./day	0.24	0.27

The active solids have an average composition of 70 per cent protein, 23 per cent carbohydrate and 7 per cent fat. In the equilibrium systems investigated, any solids in excess of the active cells were inert. The composition of the volatile solids portion of the inert sludge for the sixteen systems studied is presented in Table XXV. These values were obtained by subtraction of the active mass from the sludge analyses. Based upon the glucose carbon balances and the synthesis rates for both substrates, the active mass for the glucose systems was assumed to be equal to that for an acetate system. The active mass in a glycine system was known to be less than in an acetate system for the same load- due to the lower energy in the substrates. In lieu of long-term carbon balances data for a glycine system, the sludge in the continuous flow basin #13, which was less than in the

TABLE XXV

COMPOSITION OF INERT VOLATILE SOLIDS

Substrate	Basin	Component, percent		
		Proteins	Fats	Carbohydrates
Acetate				
Continuous Flow	1	25	5	70
Continuous Flow	2	45	5	50
Continuous Flow	5	52	6	42
Continuous Flow	6	51	8	41
Fill and Draw	3	36	4	60
Fill and Draw	4	27	6	67
Fill and Draw	7	37	4	59
Fill and Draw	8	38	4	58
Average				
Glucose				
Continuous Flow	9	41	7	52
Continuous Flow	10	37	7	56
Fill and Draw	11	48	5	47
Fill and Draw	12	46	6	48
Average				
Glycine				
Continuous Flow	13	-	-	-
Continuous Flow	14	22	7	71
Fill and Draw	15	66	4	30
Fill and Draw	16	54	7	39
Average		47	6	47

other systems, was considered to be completely active mass for the purpose of these calculations in order to make possible an approximate quantitative presentation.

The inert solids contain from one half to one third carbohydrates depending on the substrate (Table XXV). The values given for the composition of the inert volatile solids accumulated in the glycine systems should not be considered to be as reliable as those for other systems since the glycine systems contained comparatively little inert material. The inert volatile solids which would be expected to accumulate in an activated sludge system would average from 47 to 56 per cent polysaccharide with from 39 to 47 per cent protein. It is interesting to note that from 3 to 8 per cent of the inert material was measured as fats. This means that the inert material accumulating in the system contains some of each of the main constituents of the dying bacterial cells.

Although the quantitative determination of composition of the accumulating volatile solids, determined above based upon the active mass, were determined on wholly separate data from the values based upon the Long-term Carbon Balance, the percentage contents are well within the same order of magnitude. The 39 to 47 per cent protein shown above, is equivalent to 6.3 to 7.5 per cent nitrogen in the inert volatile solids. These values compare well to the 8.2 to 9.0 calculated based upon the nitrogen content of occasional grab samples of the suspended volatile solids.

In an activated sludge system, where the sludge is composed of numerous organisms, the average composition of these inert materials has by this research been shown to be quite consistent regardless of the substrate or the means by which it is fed to the system. McKinney has proposed that the bacterial slime layer is composed of polymers of amino sugars and carbohydrates which is in agreement with the results obtained in this investigation for the composition of the inert solids. It is believed that biologically inert materials are cell wall and slime layer which are residue after lysis cells.

CONCLUSIONS

On the basis of the findings in this investigation, the following conclusions have been drawn:

1. At a loading of 40 lbs of C.O.D. per 1,000 cu. ft. total capacity per day, the volatile sludge accumulates at approximately ten to fifteen per cent of the ultimate

B.O.D. of the substrate removed under equilibrium operations for waste which are carbohydrate or fatty acid in nature.

2. The rate of accumulation of volatile solids in glycine fed systems, although they follow a similar pattern to that found with other substrates, are not directly applicable to other proteinaceous wastes.

3. The accumulated sludge has been shown to be inactive mass which has accumulated because it is biologically inert to the organism normally present in activated sludge.

4. The inert volatile solids are mainly polysaccharide in nature; they do, however, contain significant amounts of fatty acids and organic nitrogen.

5. Because nitrogen is continuously removed from the active sludge in the accumulating inert material it is necessary to supplement nitrogen deficient waste with nitrogen even in uniform concentration extended sludge aeration systems operating under endogenous metabolism.

6. New sludge is being synthesized daily even in uniform concentration systems.

DEGRADATION OF NITROGEN-CONTAINING ORGANIC COMPOUNDS BY ACTIVATED SLUDGE

Interest in the biological degradability of nitrogen-containing organic compounds is twofold. First, if the nitrogen contained in the organic compound were available and sufficient for bacterial nutrient supplementation, no additional nitrogen would have to be added to promote unlimited bacterial growth. Second, as chemical and related industries continue to expand, the organic constituents of their waste streams become ever more varied. Some of these organics are nitrogen-containing; therefore, as industry attempts to reduce its pollutional load on receiving streams, it becomes important to know as much as possible about the biological degradability of nitrogen-containing organics. To deal effectively with this problem, the sanitary engineer must know how the type of nitrogen group, the number of groups, the position of the group on the organic molecule, and the structure of the organic molecule to which the nitrogen group is attached, will affect biological degradability.

Literature Survey: A very brief literature review concerning the biological metabolism of the compounds used in this study will be given. An early mention of the availability of organic nitrogen was made by Helmers, Frame, Greenberg, and Sawyer in 1951. (6) They presented the following table of organic nitrogen availability:

TABLE XXVI

AVAILABILITY OF ORGANIC NITROGEN IN VARIOUS WASTES

Waste	Per Cent Nitrogen Available at 20°C
Cotton Kiering	45
Rag-Rope	23
Brewery	72
Sewage (ave.)	73

Later Sawyer (7) in discussing his experiences at Sioux Falls, South Dakota, stated that in biologically treating a combined domestic sewage and packing-house waste there was sufficient nitrogen release from the protein degradation to cause unusually severe problems of nitrification and denitrification.

Amino Nitrogen

In 1953 Sakamoto, Uchida, and Ichinara (8) using Stanier's Method of Simulatneous Adaptation showed that o-aminobenzoic acid (anthranillic acid) was degraded through o-hydroxybenzoic acid (salicylic acid) and then to catechol. Catechol then splits into beta-keto adipic acid. Little appears in the literature concerning m-aminobenzoic, either with respect to its degradability or its method of breakdown. In 1956 Durham (9) using the Stanier Method with Pseudomonas fluorescens gave the metabolic pathway of p-mainobenzoic acid as: p-aminobenzoic acid to p-hydroxybenzoic acid to protocatechuic acid. The protocatechuic acid then breaks down to beta-keto adipic acid.

Nitro Nitrogen

Ke, Gee, and Durham (10) in 1959 demonstrated, by Stanier's technique, the breakdown of O-nitrobenzoic acid to be through o-nitrosobenzoic and then to o-hydroxylamine benzoic acid. This they likened to the suggested reduction of nitrate nitrogen to ammonia nitrogen through hydroxylamine. They also showed that cells grown on o-nitrobenzoic acid were not adapted to o-aminobenzoic acid, o-hydroxybenzoic acid, or protocatechuic acid. The only mention of m-nitrobenzoic acid breakdown found in the literature was by Cain (11) who reported in 1957 on an unsuccessful two-year attempt to culture bacteria on this substrate. In 1958 Durham (12) demonstrated by simultaneous adaptation that the first intermediate in the degradation of p-nitrobenzoic acid was p-aminobenzoic acid. Since he had already found the metabolic pathway of p-aminobenzoic acid (9), the entire breakdown of p-nitrobenzoic acid was now defined. Cain (11) disagreed with this concept, however, as he found that bacteria grown on p-nitrobenzoic acid did not adapt to p-aminobenzoic acid. Little work has been done on the poly nitro benzoic acids.

Selection of Compounds: The compounds used in this study are given in Table XXVI.

TABLE XXVI

COMPOUNDS USED IN THIS STUDY

Class	Compounds
Amino Nitrogen Compounds	o-amino Na benzoate m-amino Na benzoate p-amino Na benzoate
Nitro Nitrogen Compounds	o-nitro Na benzoate m-nitro Na benzoate p-nitro Na benzoate 3,5-dinitro Na benzoate 2,4,6-trinitro Na benzoate

Note: Nitroso nitrogen compounds were commercially unavailable and therefore could not be studied.

Procedures: In general the procedures of this investigation followed those outlined below. The activated sludges were grown in 1.5-liter pilot plants. The aeration period was 23 hours. At the end of this time the air was shut off and settling allowed to occur for one hour. At the end of the settling period, 1.0 liter of supernatant liquor was withdrawn, 1.0 liter of Cambridge, Mass. tap water replaced, organics and buffer added, and aeration resumed. This cycle was repeated seven days a week for the duration of the study. No supplemental nitrogen was added to the systems.

Investigation into the mechanism of operation of the systems was made on the Warburg Respirometer by two different types of tests. The first was a study of the possible metabolic intermediates occurring in the breakdown of the compound by the simultaneous adaptation method of Stanier's. Washed sludge, grown on the individual substrate, was placed in the Warburg Respirometer with low concentrations of several possible intermediates. The oxygen uptake was measured for six hours and at the end of that time the contents of each flask were passed through a membrane filter so that the filtrate could be analyzed for C.O.D. and the inorganic nitrogen forms. Acids were always fed as the sodium salts.

The second type of Warburg study was made by placing replicate samples of washed mixed liquor in the Respirometer flasks immediately after feeding. Then at various times during the next day a flask was removed from the Respirometer and its contents passed through a membrane filter. The filtrate was then analyzed for C.O.D. and nitrogen was then correlated to the oxygen uptake to determine the mechanism of nitrogen release.

Results:

Nitrogen Release Studies:

o-, m-, p-amino Na benzoate

The results of the nitrogen release studies for these three compounds are shown in Figures 57-59. The soluble C.O.D. and the measured and corrected net (gorss-control) oxygen uptake are also plotted on the same figures to allow ready correlation with the nitrogen data. These results are all quite similar except for the m-amino Na benzoate which will be mentioned separately. The nitrogen release was complete just as the soluble C.O.D. was removed. At the same time the oxygen uptake curves began to bend over indicating the removal of food in solution. The nitrogen was released as ammonia nitrogen; later in some cases this ammonia was oxidized to nitrite and then to nitrate nitrogen, with a corresponding increase in oxygen uptake. This oxygen uptake has been corrected for, and the net oxygen uptake for substrate oxidation only has also been plotted to show the extent of oxygen uptake due to nitrification. It takes some time for nitrifiers to develop in an activated sludge system; therefore if the nitrogen release studies are run soon enough after acclimation takes place, nitrification will not occur to any great extent. For example, in Figures 57 and 59 the sludge was only six and eight days old, respectively; therefore nitrification was small. Figure 60 shows the nitrogen release results for the o-amino Na benzoate sludge efter 81 days of operation. A comparison of this "old" sludge with the "young" sludge shown in Figure 57 demonstrates

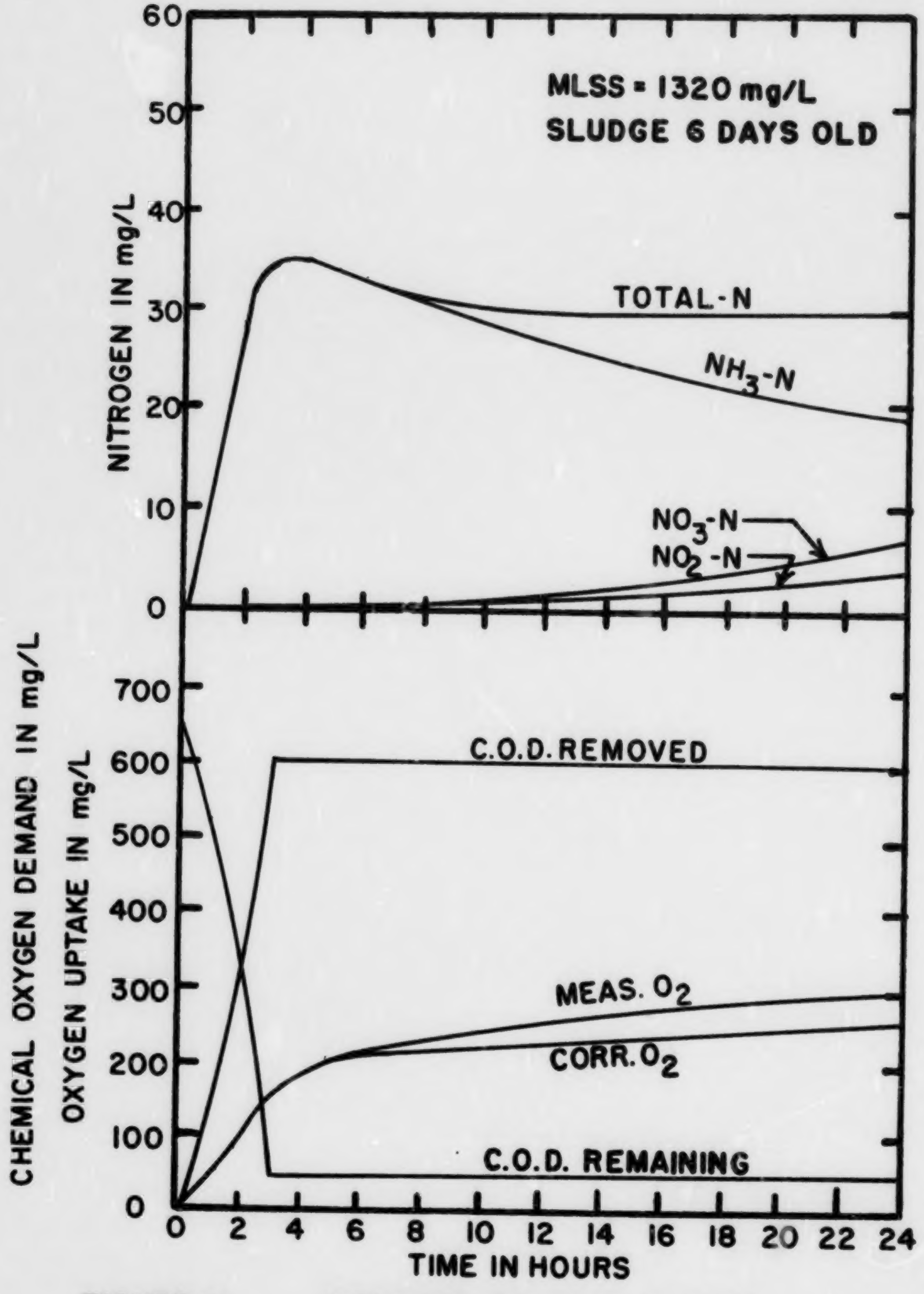


FIGURE 57 NITROGEN RELEASE STUDIES
SUBSTRATE– o-AMINO Na BENZOATE

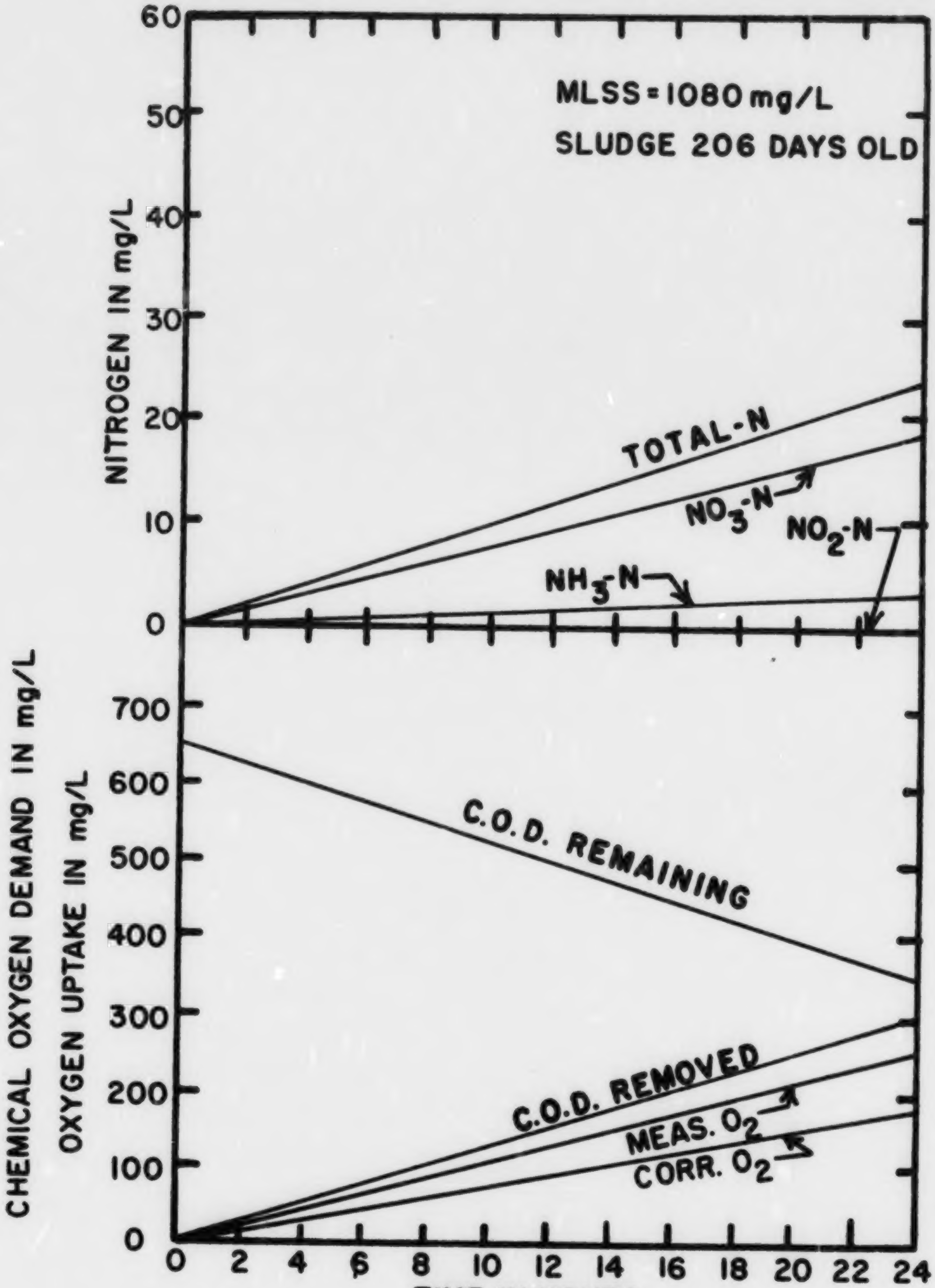


FIGURE 58 NITROGEN RELEASE STUDIES
SUBSTRATE– m-AMINO Na BENZOATE

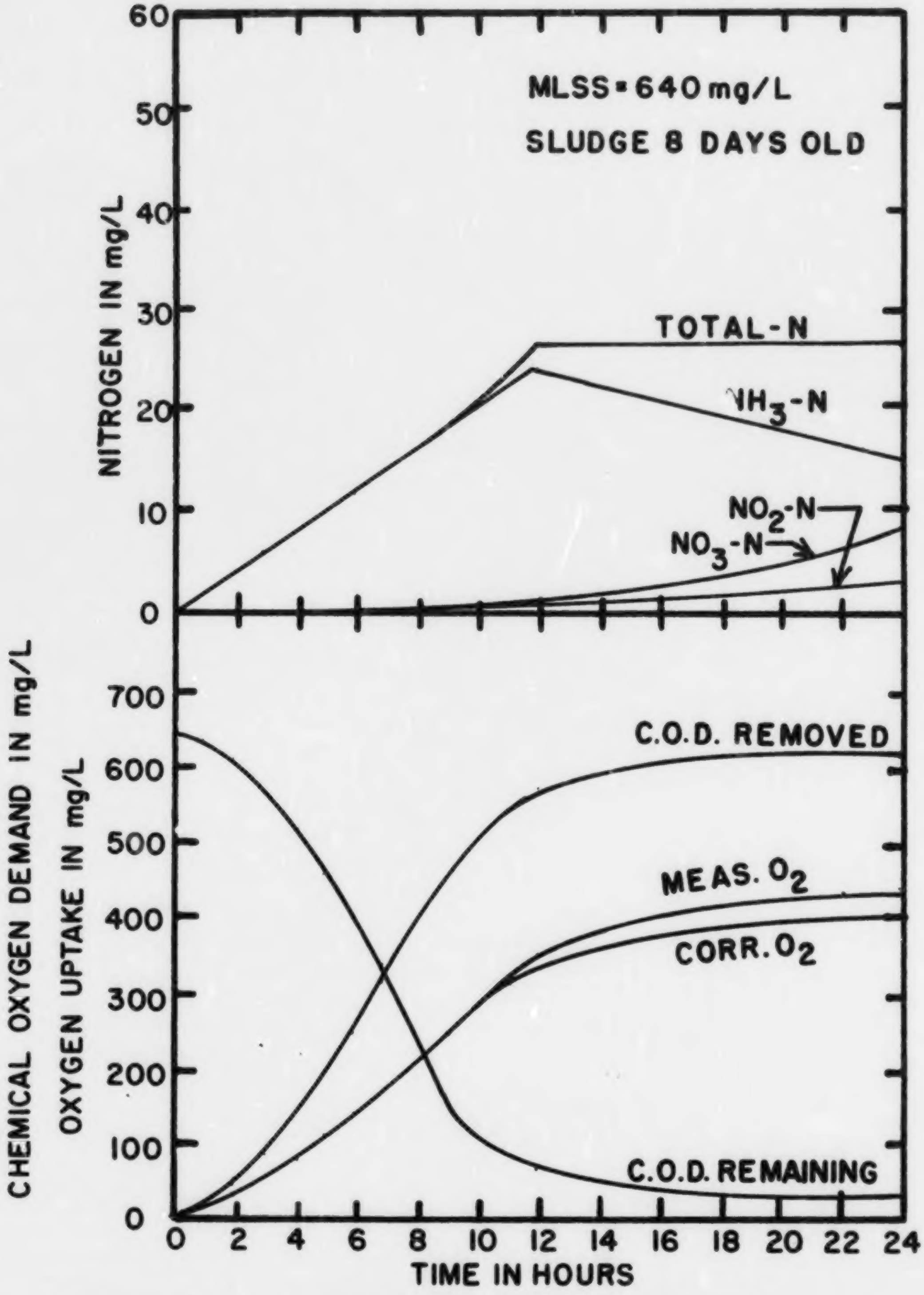


FIGURE 59 NITROGEN RELEASE STUDIES
SUBSTRATE- p-AMINO Na BENZOATE

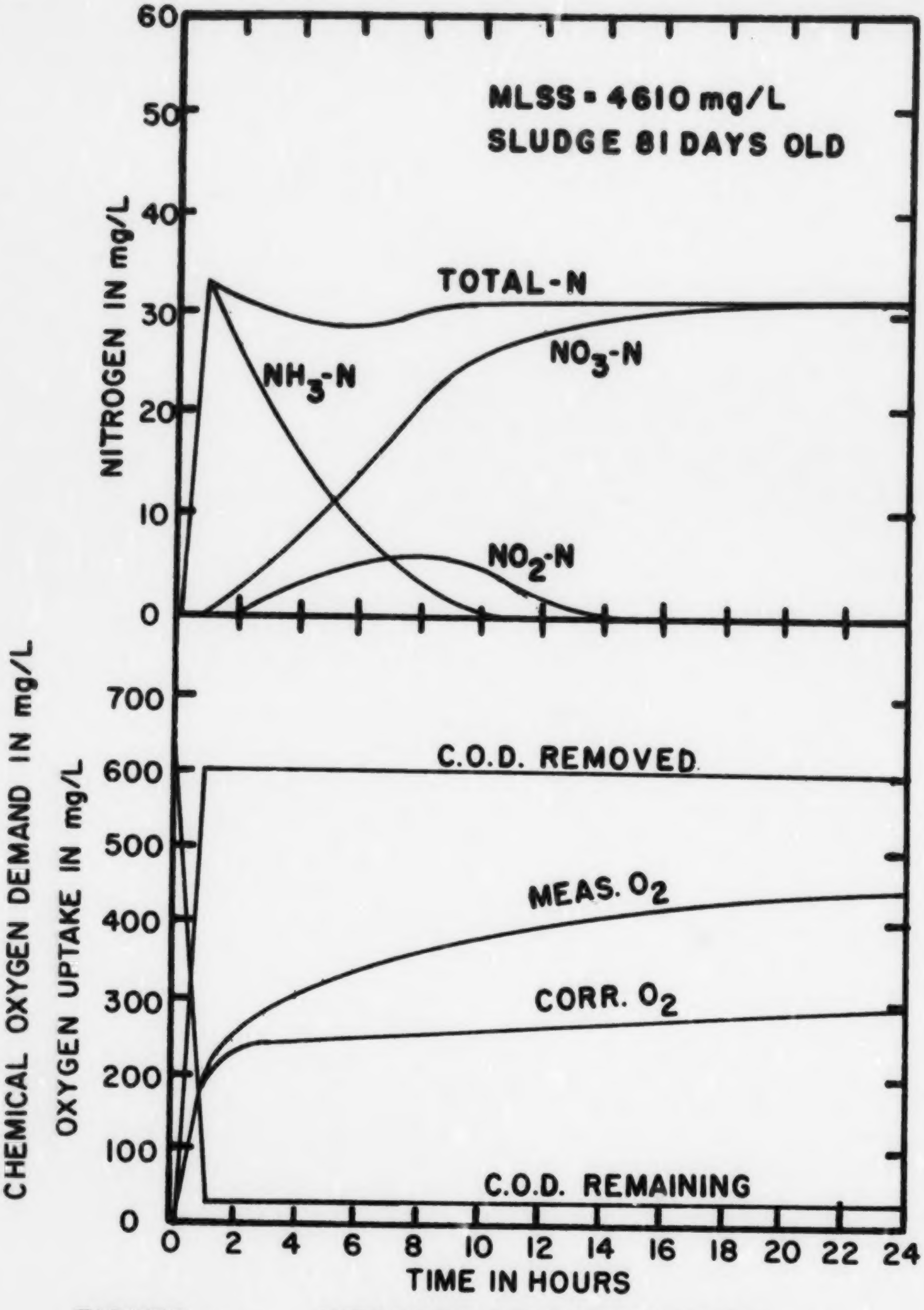


FIGURE 60 NITROGEN RELEASE STUDIES
SUBSTRATE- o-AMINO Na BENZOATE

how nitrification has become well established as the sludge aded.

Great difficulty was experienced in growing the sludge on m-amino Na benzoate. Twice during the study the sludge was discovered not to be removing 1000 mg of C.O.D. per day, and the aeration period had to be extended and then slowly shortened back to one day. Therefore, when the nitrogen release study, Figure 58,was made, the sludge was 206 days old. Prior to the run, the sludge had been operating well for 30 days and the MLSS had slowly developed to 1580 mg/L. On the day of the test, however, so many solids were lost due to the triple wash of the sludge that the MLSS fell to 1080 mg/L. At this level the sludge was able only to metabolize 338 mg/L of the added C.O.D. It would be expected that the nitrogen would be released into solution as ammonia nitrogen as occurred with the o- and p-amino Na benzoate. The nitrogen probably was released as ammonia nitrogen during the oxidation of the organic matter, but this oxidation was so slow and the number of nitrifiers so high due to the age of the sludge that the ammonia released was immediately converted to nitrates. It is hard to obtain the true mechanism of metabolism with difficult to degrade organic materials such as m-amino Na benzoate.

Nitro Nitrogen

Simultaneous Adaptation Technique

o-nitro Na benzoate

Since the report of Ke, Gee, and Durham (10) was not published at the time of these studies, it was thought that the breakdown pathway of o-nitro Na benzoate might be: o-nitrobenzoic acid to o-aminobenzoic acid to o-hydroxybenzoic acid to catechol to beta-keto adipic acid, and finally to succinic acid. In addition, protocatechuic acid was also studied. Each compound was added in such a concentration to exert a C.O.D. of 125 mg/L. The sludge was removed for testing 12 hours after feeding in an attempt to preserve the adaptive enzymes. The results of the study are shown in Figure 61. Interpretation of these results is somewhat difficult due to the problem of nitrification which can cause oxygen uptake when there is no organic degradation occurring. In an effort to avoid this problem, the extent of nitrification at the end of the run was determined and the final values of oxygen uptake corrected. The corrected data are shown in Figure 62. Since only one point, the final value, has been corrected, straight lines have been used to represent the progression of oxygen uptake for organic oxidation only. From these data it would appear that

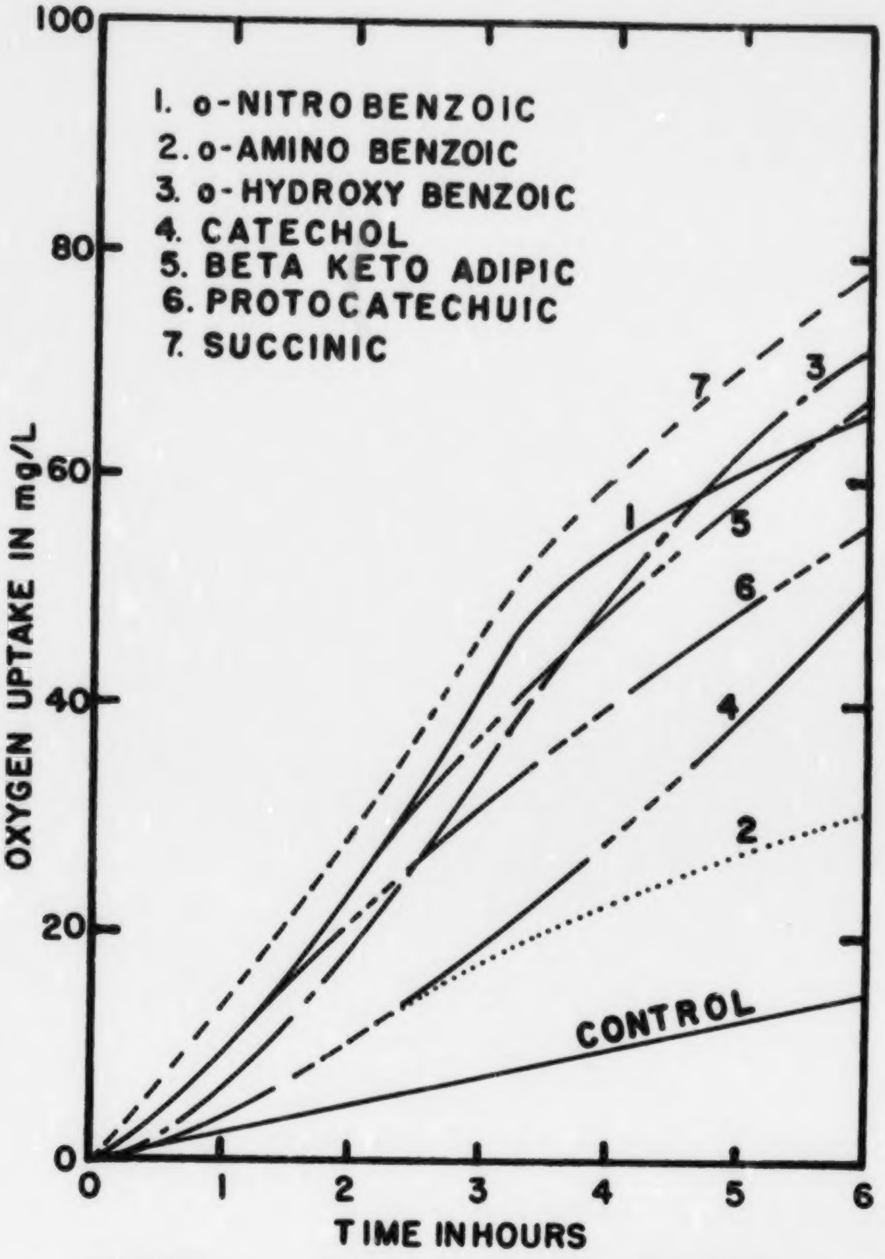


FIGURE 61 METABOLISM BY ACTIVATED SLUDGE ADAPTED TO o-NITRO Na BENZOATE UNCORRECTED FOR NITRIFICATION

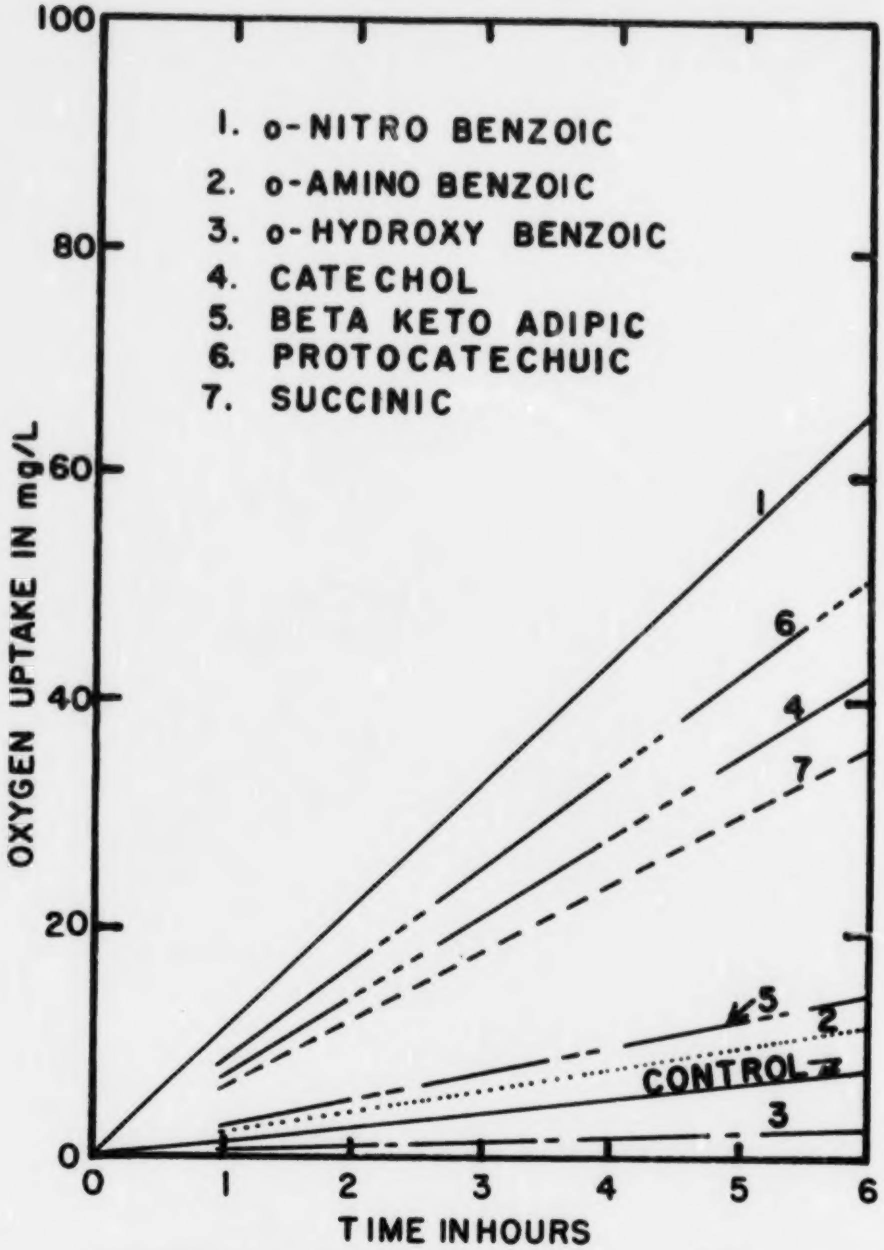


FIGURE 62 METABOLISM BY ACTIVATED SLUDGE ADAPTED TO o-NITRO Na BENZOATE CORRECTED FOR NITRIFICATION

of the possible intermediates tested, only catechol, protocatechuic acid, and succinic acid seem to be in the pathway. This confirms the work of Ke, et al. (10) that o-nitrobenzoic acid is not degraded through o-aminobenzoic acid. In their article, Ke, et al. (10) did not suggest any intermediates to follow o-hydroxylamine benzoic acid. It may well be that catechol, protocatechuic acid, and succinic acid follow o-aminobenzoic acid is in the pathway, but due to the difference in charge on the molecule, does not behave like a true intermediate when fed externally. Ke, et al. (10) did not find protocatechuic acid to be metabolized by o-nitrobenzoic acid-grown organisms, however. This pathway is still to be completely confirmed. The problem of nitrification adds still further complications to the use of Stanier's Method of Simultaneous Adaptation.

p-nitro Na benzoate

Based on the literature (9,12) the probable metabolic pathway for the breakdown of p-nitro Na benzoate was: p-nitrobenzoic acid to p-aminobenzoic acid to p-hyroxybenzoic acid to protocatechuic acid to beta-keto adipic acid to succinic acid. The compounds were added, at a concentration of 125 mg/L of C.O.D., to washed sludge which had been fed only 12 hours previously. The results of this study, corrected for nitrification (hence the straight lines) are shown in Figure 63. These data show that the first two suggested intermediates are surely in the pathway, but the last three do not seem to fit into the scheme.

3,5-dinitro Na benzoate and 2,4,6-trinitro Na benzoate

Three attempts were made to adapt a sludge to the metabolism of 3,5-dinitro Na benzoate. The first, lasting 49 days, consisted of adding 500 mg/L of C.O.D. to the unit, starting with a small sludge inoculum, and adding about 125 mg of sludge each day from a unit which was actively metabolizing p-nitro Na benzoate. After seven weeks of continual aeration, no C.O.D. reduction was noted. A second unit was started with about 1300 mg/L of sludge. This unit was fed a combination of equal parts of o-, m-, p-, and 3,5-dinitro Na benzoate daily. The total C.O.D. was 500 mg/L. After 21 days of operation the C.O.D. of the effluent showed the 3,5-dinitro Na benzoate to be undegraded. A third study consisted in continually aerating a high quantity of sludge, 1200 mg/L, with 500 mg/L of C.O.D. of 3,5-dinitro Na benzoate. After 54 days of operation, no C.O.D. reduction was noted.

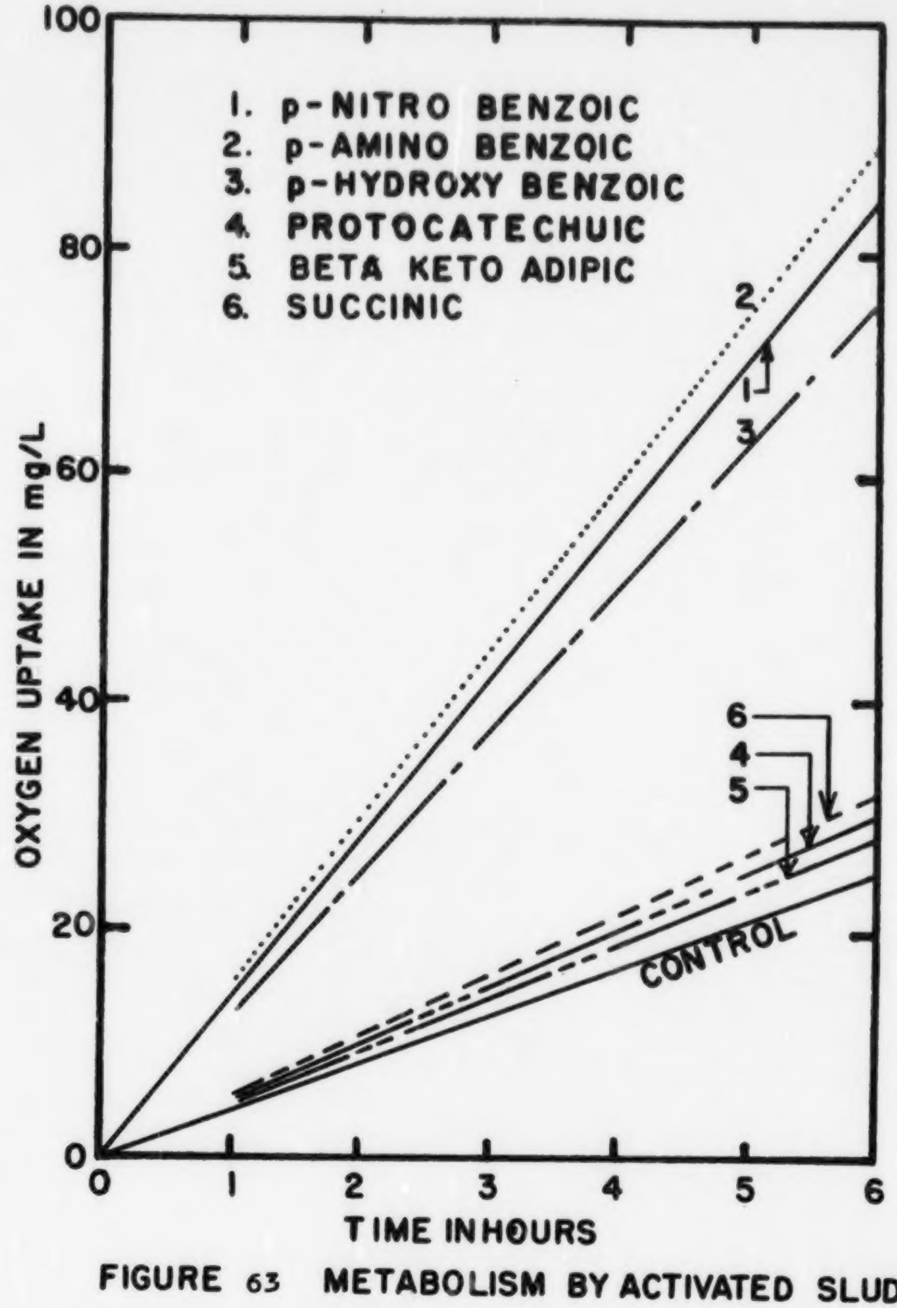


FIGURE 63 METABOLISM BY ACTIVATED SLUDGE ADAPTED TO p-NITRO Na BENZOATE CORRECTED FOR NITRIFICATION

An attempt to adapt a sludge to the metabolism of 2,4,6-trinitro Na benzoate by continually aerating a unit containing 500 mg/L of C.O.D. and daily seeding with about 125 mg of active p-nitro Na benzoate sludge was abandoned after 60 days, as no C.O.D. reduction was noted. Since neither the 3,5-dinitro Na benzoate nor the 2,4,6-trinitro Na benzoate was degradable, no simultaneous adaptation or nitrogen release studies were made. The presence of more than one nitro group on the aromatic molecule makes metabolism very difficult, if not impossible.

Nitrogen Release Studies

o-, m-, and p-nitro Na benzoate

The results of the nitrogen release studies for o-nitro Na benzoate are shown in Figure 64. This sludge was 32 days old, during which time the nitrifier population had developed enough to be converting much of the ammonia nitrogen released to nitrites and nitrates. The possibility of the nitrogen being released as nitrate and then reduced to ammonia is slight in an oxidizing environment. The internal reduction of the nitro to the amino group, followed by amino hydrolysis is a more reasonable reaction. This internal reduction supplies oxygen to the bacteria; therefore, in this case the corrected oxygen uptake is higher than the measured, taking this chemically-supplied oxygen into account.

The natural progression of partial to complete nitrification is shown by comparing Figure 64, a 32-day old sludge, with Figure 65, a 123-day old sludge. In the latter case, the nitrification reaction was so strong that no ammonia nitrogen appeared in solution at all, although it is surely produced. Interpreting Figure 66 alone might lead to the erroneous conclusion that the nitrogen is removed from nitro-containing organic compounds as nitrate instead of ammonia nitrogen.

The nitrogen release studies from the m-nitro Na benzoate-adapted sludge are shown in Figure 66. Because of the difficulty in growing a sludge which could readily metabolize 1000 mg of C.O.D. per day of this substrate, 62 days passed before the first 500 mg of C.O.D. was removed, the sludge was 126 days old when the nitrogen release studies were made. By this time nitrification was complete and the ammonia nitrogen which was probably released to solution during degradation was immediately oxidized to nitrates. Attempts to study a "young" sludge to demonstrate this ammonia release met with failure as a well-acclimated sludge could never be developed fast enough to prevent nitrification.

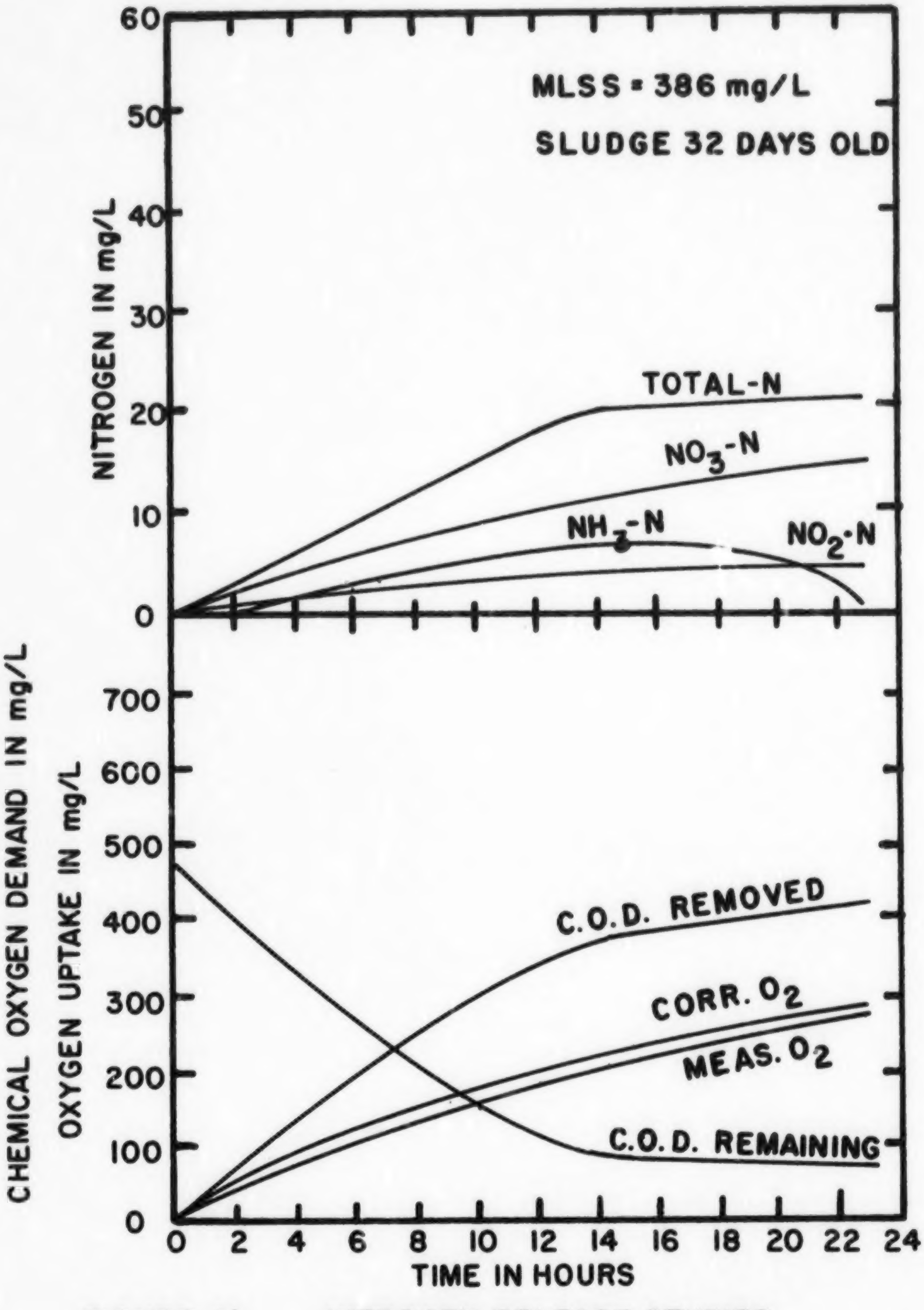


FIGURE 64 NITROGEN RELEASE STUDIES
SUBSTRATE- o-NITRO Na BENZOATE

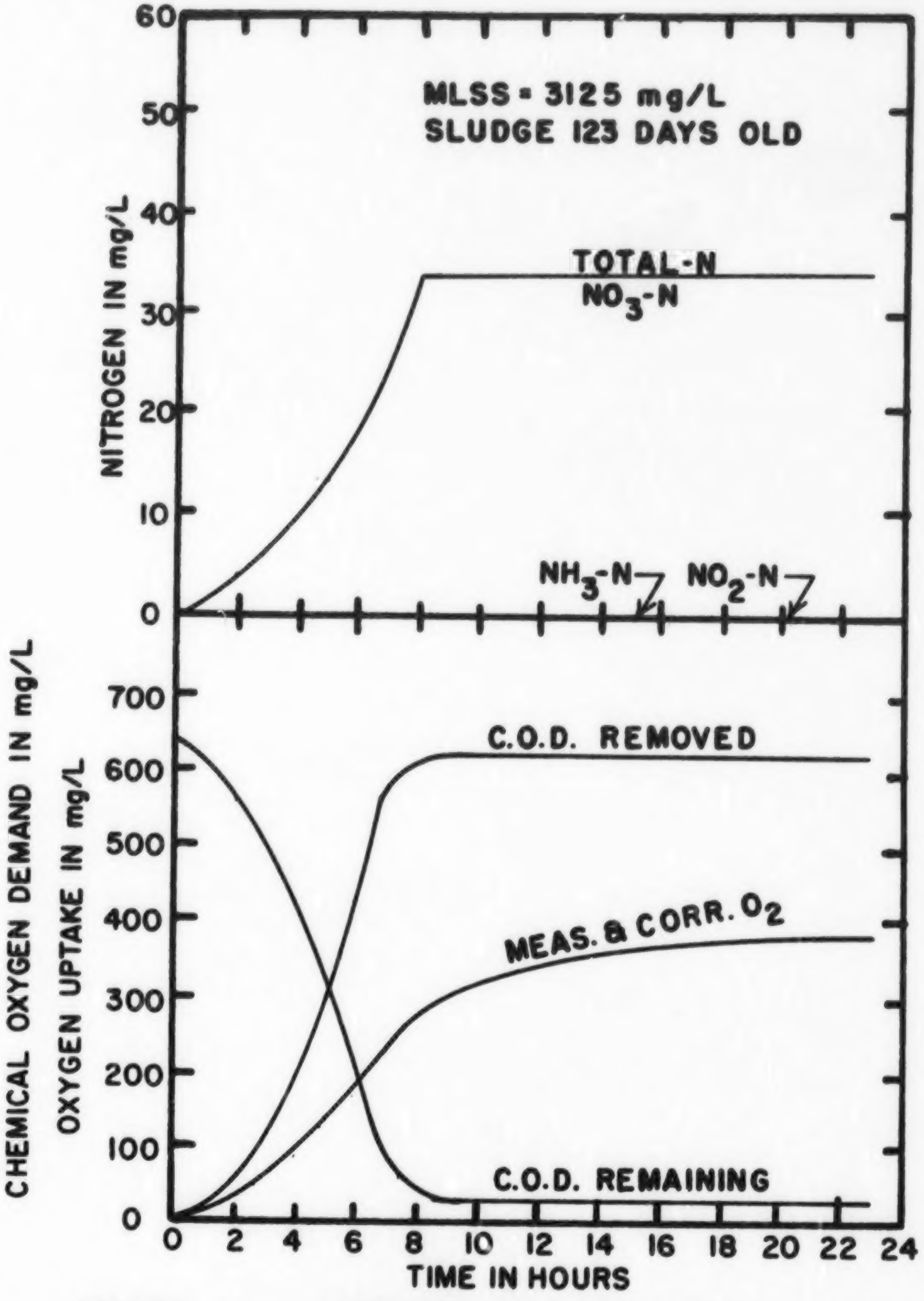


FIGURE 65 NITROGEN RELEASE STUDIES
SUBSTRATE- o-NITRO Na BENZOATE

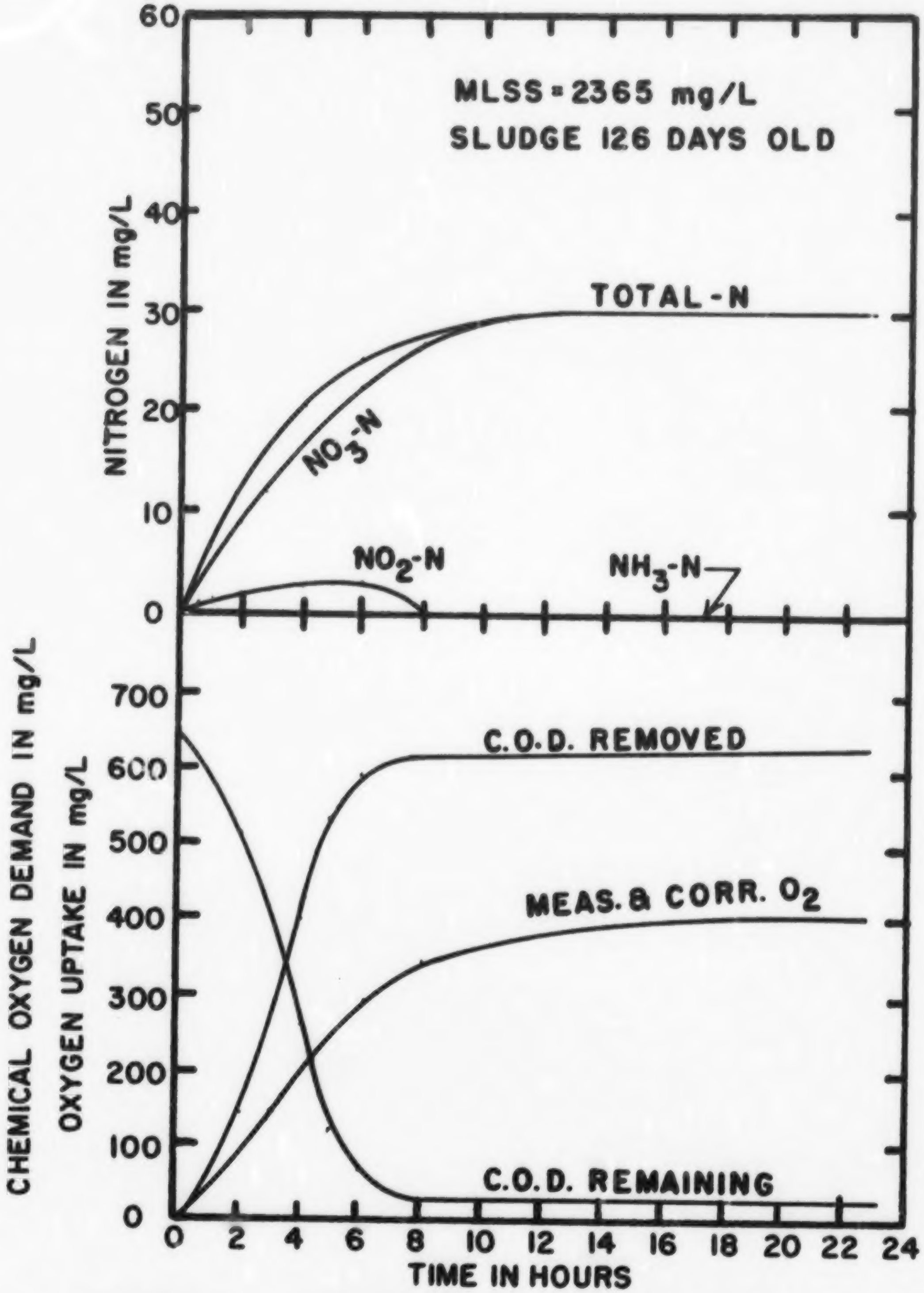


FIGURE 66 NITROGEN RELEASE STUDIES
SUBSTRATE- m-NITRO Na BENZOATE

Figure 67 shows the nitrogen release results for the sludge grown on p-nitro Na benzoate. This sludge was started with seed sludge from a unit which was already metabolizing p-nitro Na benzoate; therefore, in only 10 days this sludge was ready for study. Since this sludge was so "young" nitrification was slight and the release of ammonia nitrogen was clearly shown. The only odd feature of this study was the apparent release of all of the nitrogen before the complete removal of soluble C.O.D. A later study after 133 days of operation showed that this was an unusual condition and that after the sludge was well established the complete release of nitrogen corresponded to the complete removal of soluble C.O.D.

Discussion: Two factors hold true for all of the degradable nitrogen-containing organic compounds studied in this investigation. They are: 1) During degradation, the nitrogen contained in the molecule is released to solution and 2) the nitrogen is released as ammonia nitrogen. This means that if an activated sludge unit is to be operated on a nitrogen-containing industrial waste, and the waste is biologically degradable, the nitrogen in the molecule can be utilized for bacterial nutrient supplementation. In fact, the nitrogen in most nitrogen-containing organics is so plentiful, see Table XXVII, that some non-nitrogen-containing organics may be mixed with the nitrogen-containing waste without fear of nitrogen deficiency. The results of this investigation also show that the same overall mechanism of biological nitrogen removal, conversion to an amino group and hydrolysis from the molecule, occurs regardless of the form of the nitrogen group as it is attached to the organic molecule. This is not to say the metabolic intermediates are the same in all cases, just that the nitrogen group is removed as the amino group.

TABLE XXVII

C.O.D. TO N RATIOS OF DEGRADABLE COMPOUNDS USED IN THIS STUDY

Compound	*C.O.D./N*
mono amino benzoic acids -----------------------	16/1
mono nitro benzoic acids -----------------------	17.7/1

In order to determine the relative effect both of position and type of nitrogen group on activated sludge metabolism, the following test was made. Sludge was developed on sodium benzoate, passed through a Waring Blender and divided equally into six pilot plants. The MLSS in the six units ranged between 1324 mg/L and 1364 mg/L. To each

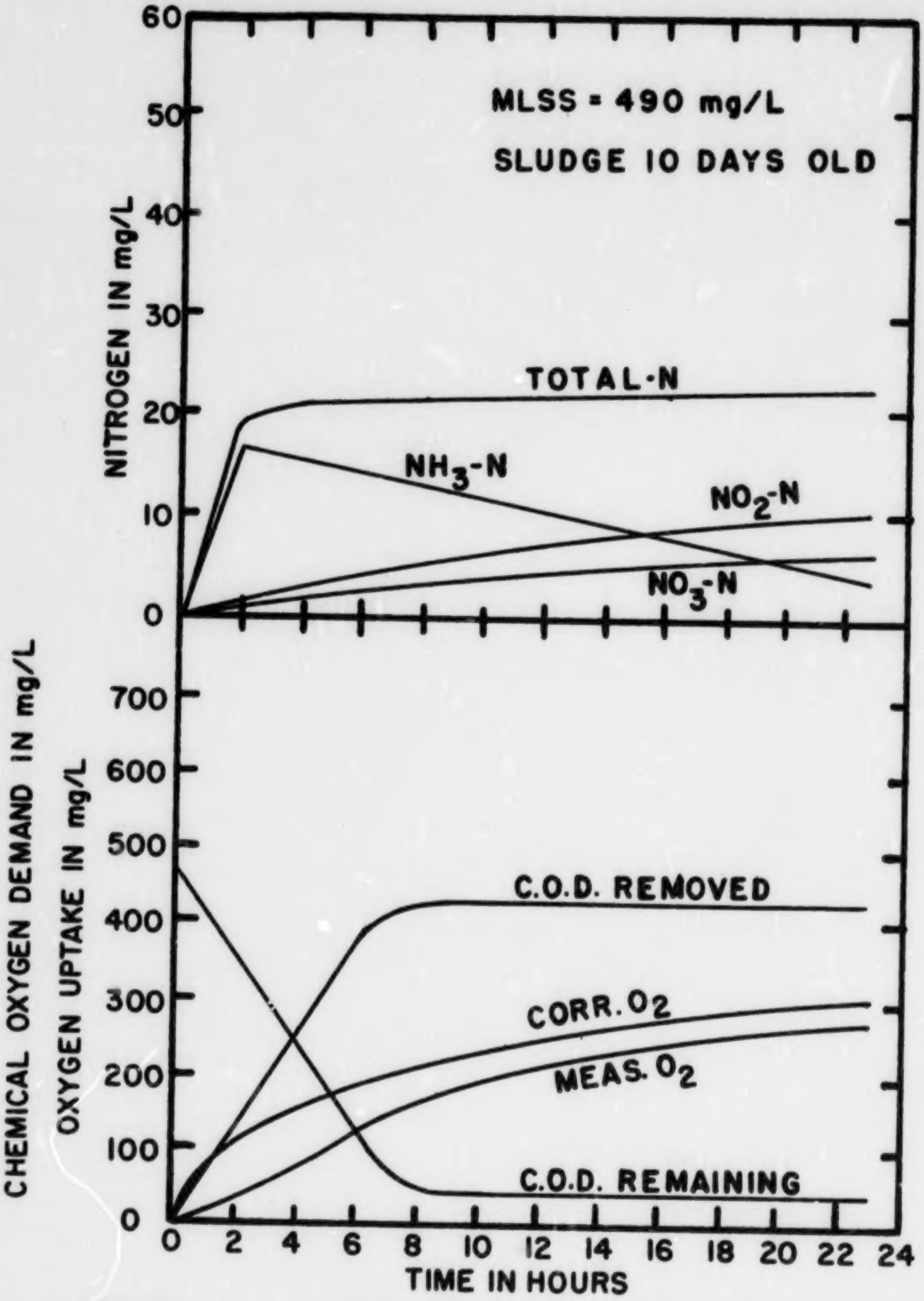


FIGURE 67 NITROGEN RELEASE STUDIES
SUBSTRATE-p-NITRO Na BENZOATE

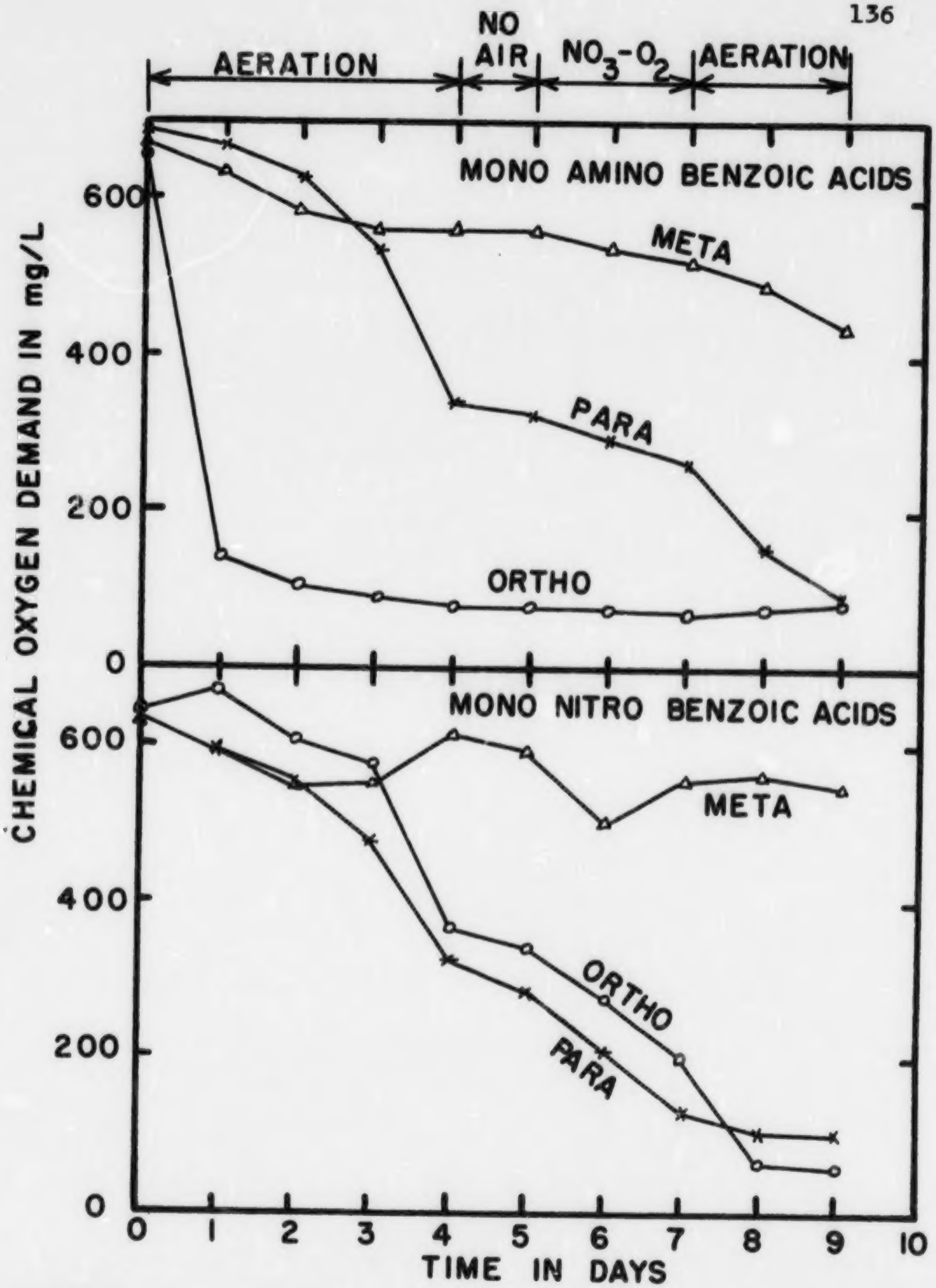


FIGURE 68 EFFECT OF NITROGEN GROUP POSITION ON METABOLISM

of these units one of the following substrates was added, o-, m-, p-amino Na benzoate, o-, m-, p-nitro Na benzoate. The initial C.O.D. concentrations were between 630 and 690 mg/L at time zero. Each day for the next nine days the soluble C.O.D. was determined in each unit. At first no nitrogen was added to any of the units. This study would then show the relative rates of metabolism of these six compounds under nearly identical environmental conditions. Unfortunately, at the end of the fourth day the air compressor failed. The units were without any oxygen for 24 hours; then, in an affort to remedy the problem, they had nitrate oxygen supplied and were stirred on a flocculator for another 48 hours until aeration was restored for the last two days of the test. The results of this test are shown in Figures 68 and 69.

In Figure 68 it is seen that in the case of the mono amino Na benzoates, the ortho position was metabolized first, then the para position and finally the meta position. With the mono nitro Na benzoates, however, the ortho and para position compounds were metabolized almost at the same rate, with the meta position compound again being the slowest. This may be explained on the basis of the nature of the groups themselves. The amino group is a small positively-charged group, while the nitro is a large negatively-charged group. The acid end of the molecule is, on ionization, negatively cahrged. In the compound o-amino Na benzoate the small amino group will be attracted to the carboxylate anion. This attraction would not be significant with the amino group in the para position. This attraction may well be the reason that the ortho compound was metabolized in one day while the para compound took much longer to be degraded.

When the nitro group is attached to the molecule the situation is quite different. If the closeness of the nitrogen group to the carboxylate anion affects biological degradability as it seems to, based on the o-amino Na benzoate data, the repulsion between the large nitro group and the carboxylate anion, when the nitro group is in the ortho position may well reduce the biological activity of the molecule due to spacial arrangement. This would explain why the o-nitro Na benzoate, which would be expected to go quite readily being an ortho compound was reduced in its activity. Meta position substitution is very hard to react, both chemically and biologically; therefore, it was not surprising that these compounds reacted slowly.

Figure 69 shows the relative rates of the ortho, meta, and para positioned groups, comparing in each case the amino and nitro group. In the case of the ortho and meta positions,

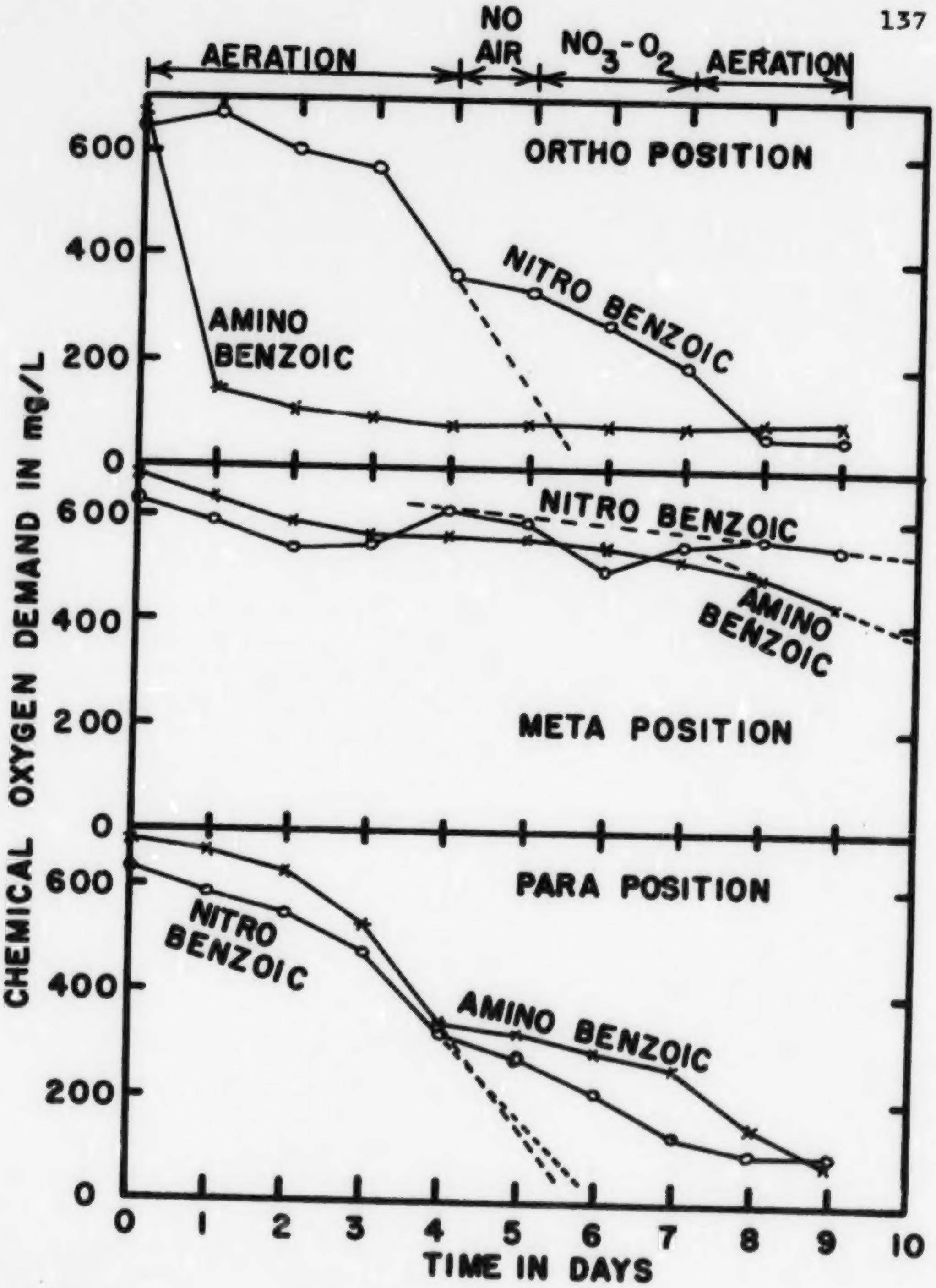


FIGURE 62 EFFECT OF NATURE OF NITROGEN GROUP ON METABOLISM

the amino group was more readily attacked than the nitro group. This was probably the case in the para position also, except that the interruption of aeration strongly affected the rate of p-amino Na benzoate metabolism. The projected curves (dotted lines) of Figure 69 show the p-amino Na benzoate unit reached the 100 mg/L C.O.D. level about 0.3 days before the p-nitro Na benzoate unit reached the same level. In any event, the difference in group had the least effect in the para position, possible because the group was so far from the carboxylate anion as to exert little influence on the spacial arrangement of the molecule, thereby exerting little influence on the biological degradability of the compound. Spacial arrangement seems to have an important effect on bacterial metabolism. Based on the projected curves in Figure 69, Table XXVIII gives the relative rates of compound degradation,

TABLE XXVIII

TIME TO REACH 100 mg/L LEVEL OF C.O.D. BASED ON PROJECTED CURVES

Compound	Time To Be Degraded, in Days
o-amino Na benzoate	2.8
p-amino Na benzoate	5.2
o-nitro Na benzoate	5.2
p-nitro Na benzoate	5.5
m-amino Na benzoate	16.5
m-nitro Na benzoate	46

Conclusions: Based on the results of this investigation, the following conclusions may be drawn:

1. o-, m-, and p-amino Na benzoate, o-, m-, and p-nitro Na benzoate were all found to be biologically degradable by activated sludge.
2. 3,5-dinitro Na benzoate and 2,4,6-trinitro Na benzoate were not biologically degradable by activated sludge.
3. The nitrogen-containing organic compounds which were biologically degradable released their nitrogen into solution during metabolism. Once in solution, this nitrogen could be used for synthesis.
4. The meachanism removal of nitrogen from the organic molecule was the same regardless of the original form of the nitrogen group. The nitrogen group was converted

to an amino group, if necessary, and was then hydrolysed from the molecule as ammonia nitrogen.

5. Both the form, position, and number of nitrogen groups has an important influence on the ease of biological degradability of an organic compound containing nitrogen.

6. Metabolism of p-nitro Na benzoate by activated sludge appears to be by reduction of the nitro group to form p-aminobenzoic acid which is then converted to p-hydrobenzoic acid. Beyond this point the pathway was not defined.

7. Care must be taken in the interpretation of results from Stanier's Method of Simultaneous Adaptation because of problems with nitrification, toxicity, and chemical reactivity of the suspected intermediate. Also compounds which are formed as intermediates intracellularly may have difficulty diffusing across the cell wall when fed externally due to differences in charge.

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APPENDIX

DETAILED EXPERIMENTAL PROCEDURES

A) General Operation of Pilot Units

The continuous flow pilot units received 1070 mg of oxidizable organics per day in the form of sodium acetate, glucose or glycine. This gives a loading of 60 lbs. of oxidizable organics/1000 cu. ft. of aeration capacity, which is the loading of several successful field units. These three substrates will represent the three general classes of organic compounds, fats, carbohydrates, and proteins. The organics are dissolved in 1.7 liters of water as this is the daily capacity of the pump. The hydraulic detention time in the aeration section is 14 hours but the organic matter is being fed at the rate of 44 mg. of C.O.D./hr. The substrate is buffered and contains ample nitrogen. The system is buffered at pH 6.8-7.0 to cause the release of the formed CO_2 and prevent the buildup of alkalinity. The effluent is discharged into a two liter bottle under pressure. Samples for analysis are stored at 4°C.

The operation of the fill and draw system differs somewhat from the continuous flow system. The fill and draw unit is aerated for 23 hours, the air turned off, the mixed liquor allowed to settle for one hour, 1.0 liters of effluent removed, 1.0 liters of substrate replaced and aeration resumed. This unit receives exactly the same quantity of organic matter, buffer and nitrogen as the continuous flow unit. Effluent samples are composited over the test period and stored at 4°C for analysis.

B) Carbon Balance

In order to determine when the systems being studied have reached steady conditions with respect to per cent oxidation and synthesis of the substrate removed, a complete carbon balance was made on the systems. Everything entering and leaving the limits was measured as carbon. The per cent of removed carbon, collected as CO_2, was a measure of the oxidation. The closing error of the balance was a measure of the overall accuracy of the method. Carbon balances were attempted on small test tube cultures, one-day balances in the pilot units, and one-week balances and two-week balances in the pilot units. The random analytical errors in the measurements made the closing errors unreasonably large in the test tube and one-day balances. Extending the time period to one week greatly improved the accuracy, and making a two-week balance further reduced the closing error.

The one or two-week carbon balances are made as follows. The chemical oxygen demand is measured in triplicate on the feed solution. This determines the amount of C.O.D. added to each unit. This determination is repeated every time a new stock feed bottle is needed. The C.O.D. of the feed is converted to mg of carbon by multiplying by the appropriate factor.

During the test, 50 ml per day of effluent was composited in a storage bottle for analysis at the end of the test. This gives a measure of the average effluent quality during the test period. At the end of the test, if possible, 200 ml of this composite effluent is passed through a membrane filter and the total and volatile solids are measured. The volatile solids are ignited in a covered crucible at 700°C. The total weight of volatile solids discharged during the test period are calculated on the basis of the total volume of effluent discharged. This weight is converted to carbon on the basis of the per cent carbon in the sludge as determined by periodic microchemical analyses. This factor varied from 0.45 to 0.50.

The membrane filtrate of the effluent suspended solids determination is measured for C.O.D. to determine what organic matter is descharged from the units during the test period. This is converted to total mg of carbon lost during the test period by multiplying the same factor as the feed. This assumes that the discharged organic matter is unutilized feed. This is probably not true, but the nature of the organics causing the C.O.D. in the effluent is not exactly known. The amount of carbon involved was small, however.

The weight of volatile solids in the mixed liquor was measured at the end of each test period by the membrane filter technique. This measure was made in duplicate to increase the accuracy. Before this test was made the entire contents of both the continuous flow and fill and draw system were thoroughly mixed to allow calculation of the weight of volatile sludge in the entire basin by multiplying the concentration in mg/L by 1.5. This weight of cells was converted to carbon by the same microchemical analysis mentioned above. The amount of carbon as cells removed from the basins during these analyses were also taken into account in the balances.

The membrane filtrate of the mixed liquor suspended solids determination was analysed for C.O.D. to determine the organic matter in solution in the basins. This was converted to carbon by multiplying by the same factor used for the feed. Again the assumption that this C.O.D. unutilized food is probably not true, but again the figures were small and the error introduced not too great.

The evolved CO_2 was caught in two traps, one of 1.oL capacity and the other 500 ml. Less than 10% of the total was found in the second trap. Before analysis the traps are made up to known volume. Fritted glass bottom crucibles were cleaned, heated, and weighed. Ten ml of 1 N $BaCl_2$ was heated in a test tube in boiling water to speed the reaction and 10 ml from the trap was added. This mixture was shaken, and filtered under a nitrogen atmosphere into a suction flask. A nitrogen atmosphere was provided by holding an inverted funnel connected to a compressed nitrogen tank over the filter set-up.

The filtered $BaCO_3$ was washed four times with a little distilled water to remove $BaCl_2$. The crucible was dried at 103°C and the final water removed at 700°C. The $BaCO_3$ does not convert to BaO until 1450°C is reached so the sample was weighed as $BaCO_3$. The CO_2, if any, in the 1N NaOH used in the traps, and the CO_2 picked up from the atmosphere during the testing procedure was determined by the use of a blank. This balnk was 10 ml of the 1N NaOH used in the traps, and was carried through the same procedure as the samples. This value was subtracted from the readings. The $BaCO_3$ was converted to weight of carbon by multiplying by 0.0603.

No alkalinity measurements were made, as at pH 7.0 the total alkalinity was only about 4 mg/L, and at pH 6.0 is only about 0.4 mg/L, both of which were insignificant.

Carbon balances were made by comparing total carbon removed during the test with the total carbon accounted for in the CO_2 evolved and cells formed. The per cent oxidation of the system was the carbon recovered as CO_2 divided by the total carbon removed.

C) Separation of Compounds

In order to find out what compounds are being synthesized under the established environmental conditions and at what rate these compounds are degraded under non-fed conditions it was essential to take samples of the bacterial mass at various time periods and then fractionate these samples into pure fractions of proteins, fats, and carbohydrates. It is also important to be able to measure the amount of material in each of these fractions.

a) Proteins

1) Separation

A 10-20 sample of mixed liquor was refluxed for 20 hours with 6N HCl. This was evaporated to dryness twice to remove the HCl. Water was added, the pH was now about 2.0. The mixture was then shaken with 1.5 gms. of activated carbon. This removed the fats and carmlized sugars. This mixture was then filtered and the filtrate, containing the protein derivatives, was evaporated nearly to dryness and then transferred to the Van Slyke combustion tube for final drying.

2) Measurement

The nitrogen was measured on a duplicate untreated sample of sludge by Kjeldahl nitrogen. Data taken indicate that 90 per cent of the nitrogen contained in bacterial cells was measured by Kjeldahl nitrogen. Investigation of the literature showed the average percentage of proteins was 45 per cent and nucleic acids 20 per cent in bacterial cells. The average per cent nitrogen in proteins is 16 per cent and 22 per cent in nucleic acids. Combining these figures,

$$90\frac{(0.16 \times 0.45 + 0.22 \times 0.20)}{0.66} = 16.2 \text{ per cent.}$$

This means a multiplying of 6.2 will convert the Kjeldahl nitrogen to protein plus nucleic acids.

b) Fats

1) Separation

A 50 ml sample of mixed liquor was evaporated to a 40 ml volume and combined with 40 ml of concentrated HCl, digested for 1 hour on the steam bath, and evaporated to dryness to remove some HCl. The residue was dissolved in a small portion of distilled water and absorbed into filter paper. The filter paper was placed in an extration thimble and dried for 15 minutes. The sample was then extracted in a Soxhlet apparatus for nine hours with petroleum ether. None of the carmelized sugars or much of the nitrogen containing material was soluble in the petroleum ether. The extracted sample was evaporated on the steam bath almost to dryness and then transferred to the Van Slyke combustion tube for final drying.

2) Measurement

Another 100 ml sample was carried through the extraction procedure and the sample was dried on the steam bath almost to dryness. The flask was then inverted and dried in a desiccator under vacuum. Air was then blown into the flask to remove the last traces of either in the flask before weighing. This prevents the loss of fats due to evaporation to dryness on the steam bath and heating at 103°C in the oven. The flask was then weighed to obtain the quantity of fats.

c) Carbohydrates

1) Separation

A 25 ml sample of mixed liquor was refluxed for 2 hours with 0.1N HCl. This converts the carbohydrates to glucose. The sample was then neutralized to pH 6.4 and admium sulfate added to precipitate non-glucose reducing materials as well as the proteins and fats. The sample was then filtered and the filtrate demineralized by passing it over 75 ml of a monobed ion exchanger, Amberlite MB-3. The demineralized filtrate was then evaporated almost to dryness and transferred to a Van Slyke combustion tube for final drying.

2) Measurement

Another 25 ml sample was carried past the filtration step after the cadmium sulfate precipitation and the glucose in the filtrate was measured by a modification of the Somogyi procedure for reducing sugars. By this procedure the reducing sugars were oxidized by a standard $CuSO_4$ plus KIO_3 solution by heating for a definite period of time under alkaline conditions. The remaining oxidizing reagent was used to liberate iodine from KI under acid conditions. The evolved iodine was measured by standard sodium thiosulfate. The difference between the blank and the sample titration was a measure of the reducing sugar content of the sample.

D) Radioactive Studies

a) Operation of Units

The radioactive studies were made with sodium acetate with the C^{14} in the C_1 position, C_2^{14} sodium acetate, C_u^{14} glucose, and C_1^{14} glycine. When the carbon balances indicate the units have reached steady conditions the units were sampled and the proteins, gats, and carbohydrates are determined as outlined above. One week later this testing was repeated as a check of the values. The next day the batch unit was dosed with 0.019 mc of radioactive feed plus the normal amount of ordinary feed. The continuous flow system is fed for two hours at the usual organic concentration. During that time 0.012 mc of radioactive feed was added directly in eight equal doses at 15 minute intervals. This technique avoided loss of any radioactivity through the feed mechanism.

At regular time intervals such as 0, 1/4, 1/2, 3/4, 1, 1-1/4, 1-1/2, 1-3/4 and 2 hours, and 1, 2, 3, 4, 7, 14, 21 days, 80 to 100 ml samples were withdrawn from both basins for separation and counting. Sample size was adjusted to allow enough carbon to be in each fraction to produce an infinitely thick sample for counting. This proceudre yields the rate of production of the three major components of bacterial cells, during synthesis, and the rate of breakdown when feeding ceases. Samples were taken on duplicate basins.

b) Combustion of Samples

After the samples have dried at 103°C in the combustion tubes they are burned by the use of Van Slyke's Reagent and a bunsen flame. The evolved CO_2 is absorbed in the 1N NaOH trap, the $BaCl_2$ and H_2O traps being used as safety factors. Five times the required NaOH was placed in the first test tube.

c) Counting of Samples

One ml of 1N $BaCl_2$ was placed in the NaOH trap and the $BaC^{14}O_3$ precipitated out. One ml was insufficient to precipitate all the $BaC^{14}O_3$ so that the same amount of precipitate is collected each time. This material was passed through a 20 mm filter, S and S No. 895, was washed once with distilled water and once with acetone to improve drying. The sample was then air dried and counted for 400 counts. This gave a coefiicient of variation of 5 per cent. This plating procedure should give an infinitely thick sample to avoid corrections due to self-absorption. Any samples which looked thin were weighed as a check.

A special sample holder was designed and constructed which placed the sample very close to the window of the counter to avoid loss of counts due to a large air space between sample and window.